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Role of AKT/ PKB and 14-3-3 in the Regulation of B Cell Receptor Signaling and Signalosome Assembly

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Role of PKB/AKT and 14-3-3 in the Regulation of B Cell Receptor Signaling and Signalosome Assembly

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Stockholm 2015

*“My pain... may be the reason for somebody's laugh but
My laugh... must never be the reason for somebody's pain”*

Charlie Chaplin (1889-1977)



Dedicated to

KURDISTAN

(My dream country)

*“As I walked out the door toward my **FREEDOM** I knew that if I did not leave all the anger, hatred, and bitterness behind, that I would still be in **PRISON**”*

Nelson Mandela (1918-2013)



Thesis defense

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ABSTRACT

AKT/PKB is an oncogenic serine/threonine kinase regulated via the PI3K pathways. 14-3-3s represent a large group of adaptor proteins that are known to interact with a plethora of signaling proteins and regulate diverse signal transduction pathways. The B-cell antigen receptor (BCR) activation and signalosome assembly are dynamic processes controlled by protein phosphorylation. The signaling events share their functions in controlling cell proliferation, differentiation, and/or apoptosis.

In Paper I, the first characterized protein is 14-3-3 ζ , which was found to be a new regulator of BTK. Two 14-3-3 ζ binding-sites were found to be phosphorylated by AKT/PKB and mapped to phospho-serine pSer51 in the PH domain and to phospho-threonine pThr495 in the kinase-domain. The PI3K inhibitor LY294002 abolished S51/T495 phosphorylation and disrupted the interaction. Moreover, inhibitors targeting 14-3-3 (BV02) and BTK (Ibrutinib) compromised interaction between the two proteins. Nuclear translocation of BTK was promoted following down regulation of 14-3-3 ζ . Furthermore, the loss-of-function mutant S51A/T495A displayed reduced tyrosine-phosphorylation and inability to bind to 14-3-3 ζ . Conversely, the gain-of-function mutant S51D/T495D exhibited intense phosphorylation, enhancing interaction of BTK with 14-3-3 ζ . Phosphorylation of this mutant was associated with ubiquitination and degradation of the protein, presumably, contributing to the termination of the B-cell receptor signaling.

In Paper II, we identified a new BTK-partner, ankyrin repeat domain 54 protein (ANKRD54) that binds to the BTK SH3 domain. Our results suggest that ANKRD54 specifically mediates nuclear export of both BTK and another TEC family kinase member, TXK/RLK. The interaction site was mapped to the C-terminus of the BTK SH3 domain, since a synthetic peptide covering this region, ARDKNGQEGYIPSNYVTEAEDS, was sufficient for mediating this interaction. ANKRD54 is the first protein reported to specifically influence nucleo-cytoplasmic shuttling of BTK. ANKRD54 probably belongs to a novel group of proteins carrying out this activity in a Crml-dependent manner.

In Paper III, using proteomics, we identified 446 proteins, containing 186 novel AKT-associated-motif (RXRXXS/T) phosphorylation events. B-cell receptor induction leads to up regulation of 85 proteins and down regulation of 277 proteins. Proteins related to ribosome biogenesis, DNA binding, transcription and translation regulation were mainly up regulated. Conversely, down regulated proteins were mainly involved in RNA binding, mRNA splicing and mRNP export. Immunoblotting of two proteins RBM25 and MEF-2D were positively validated in the mass spectrometry data. Consistent with these findings, the AKT-inhibitor (MK-2206) remarkably reduced phosphorylation of the target proteins on the RXRXXpS/T motif, while the mTORC2-inhibitor (PP242) totally blocked this phosphorylation.

In Paper IV, we found that AKT/PKB induces BLNK and SYK phosphorylation, which promotes the 14-3-3 binding in a Ser/Thr phosphorylation-dependent manner. Using an *in vitro* phosphorylation screening assay, we identified BLNK and SYK as excellent substrates of AKT. Moreover, the AKT/PKB inhibitor MK2206 reduced phosphorylation of BLNK and SYK. Additionally, 14-3-3 regulates the stable interaction between SYK and BLNK and sustains phosphorylation of SYK and BLNK. Furthermore, 14-3-3 compromises binding of SYK to Importin 7 thereby abrogating shuttling of the protein to the nucleus. Alanine substitutions of T256, S295 or S297 sites resulted in abrogation of SYK binding to Importin 7. Interestingly, BLNK phosphorylation at Y84 appears to correlate with the degree of tyrosine phosphorylation of SYK at position(s) Y525/526.

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LIST OF PUBLICATIONS

- I. **DARA K. MOHAMMAD**, Beston F. Nore, Alamdar Hussain, Manuela O. Gustafsson, Abdalla J. Mohamed and C. I. Edvard Smith. Dual phosphorylation of BTK by AKT/PKB Provides Docking For 14-3-3 ζ , Regulates Shuttling and Attenuates both Tonic and Induced Signaling in B Cells. (2013) *Molecular and Cellular Biology* 33, 3214-3226
- II. Manuela O. Gustafsson, Alamdar Hussain, **DARA K. MOHAMMAD**, Abdalla J. Mohamed, Vivian Nguyen, Metalnikov, P., Colwill, K., Tony Pawson, C. I. Edvard Smith and Beston F. Nore. Regulation of nucleocytoplasmic shuttling of Bruton's tyrosine kinase (BTK) through a novel SH3-dependent interaction with ankyrin repeat domain 54 (ANKRD54). (2012) *Molecular and Cellular Biology* 32, 2440-2453
- III. **DARA K. MOHAMMAD**, Hashim A. Raja, Janne J. Turunen, Beston F. Nore and C. I. Edvard Smith. B Cell Receptor (BCR) Activation Predominantly Regulates AKT Associated Motif Phosphorylation in Proteins Related to RNA Processing. (Manuscript)
- IV. **DARA K. MOHAMMAD**, Beston F. Nore, Manuela O. Gustafsson, Abdalla J. Mohamed and C. I. Edvard Smith. AKT/PKB Attenuates SYK and BLNK through 14-3-3 Impairing Nuclear Translocation of SYK via Importin 7 in B Cells. (Submitted)

Publications and manuscripts by the author not included in the thesis:

- I. Alamdar Hussain, Liang Yu, Rany Faryal, **DARA K. MOHAMMAD**, Abdalla J. Mohamed and C. I. Edvard Smith. TEC family kinases in health and disease loss-of-function of BTK and ITK and the gain-of-function fusions ITK-SYK and BTK-SYK. (2011) *The FEBS Journal* **278**, 2001-2010
- II. Alamdar Hussain, **DARA K. MOHAMMAD**, , Manuela. O. Gustafsson, Merve Uslu, Abdulrahman Hamasy, Beston F. Nore, Abdalla J. Mohamed and C. I. Edvard Smith. Signaling of the ITK (interleukin 2-inducible T cell kinase)-SYK (spleen tyrosine kinase) fusion kinase is dependent on adapter SLP-76 and on the adapter function of the kinases SYK and ZAP70. (2013) *The Journal of Biological Chemistry* **288**, 7338-7350
- III. Burcu Bestas, Pedro Moreno, Emelie M. Blomberg, **DARA K. MOHAMMAD**, Amer F. Saleh, Tolga Sutlu, Joel Z. Nordin, Peter Guterstam, Manuela O. Gustafsson, Shabnam Kharazi, Barbara Piatosa, Thoma C. Roberts, Mark A. Behlke, Mathew J. A. Wood, Michel J. Gait, Karin E. Lundin, Samir El Andaloussi, Robert Månsson, Anna Berglöf, Jesper Wengel and C. I. Edvard Smith. Splice-correcting oligonucleotides restore BTK function in X-linked agammaglobulinemia model. (2014) *The Journal of Clinical Investigation* **124**, 4067-4081
- IV. Dina Ali, **DARA K. MOHAMMAD**, Huthayfa Mujahed, Kerstin Jonson-Videsäter, Beston F. Nore, Christer Paul and Sören Lehmann. Anti-leukemic effects of APR-246 can be enhanced by inhibiting the protective response of the Nrf2/HO-1 pathway through inhibition of PI3K and mTOR in AML cells. (Submitted)
- V. Alamdar Hussain, Abdulrahman Hamasy, **DARA K. MOHAMMAD**, Manuela O. Gustafsson, Beston F. Nore, Abdalla J. Mohamed and C. I. Edvard Smith. Role of N-terminal region in the regulation of SYK-fusion kinases ITK-SYK, BTK-SYK and TEL-SYK. (Manuscript)

LIST OF ABBREVIATIONS

ABL	Abelson murine leukemia viral oncogene homolog
ALL	Acute lymphoblastic leukemia
ARM	Armadillo motifs
ANKRD54	Ankyrin repeat domain 54
BCL	B cell lymphoma
BCR	B cell receptor
BASH	B cell adaptor containing an SH2 domain
BCR	Breakpoint cluster region
BCAP	B cell cytoplasmic adaptor protein
BLNK	B cell linker protein
BM	Bone marrow
BMX	Bone marrow tyrosine kinase in chromosome X
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
CBL	Casitas B-lineage lymphoma
DAG	Diacylglycerol
DCs	Dendritic cells
GFP	Green fluorescent protein
GRB2	Growth factor receptor-bound protein 2
HSCs	Hematopoietic stem cells
HPLC	High performance liquid chromatography
IBB	N-terminal Importin β binding domain
IL-	Interleukin
IP3	Inositol (1,4,5)-trisphosphate
ITAM	Immunoreceptor tyrosine based activation motif
ITK	IL-2 inducible T cell kinase
KO	Knockout
LY294002	2-(4-morpholinyl)-8-phenylchromone
MEF-2D	Myocyte-specific enhancer factor 2D
NFAT	Nuclear factor of activated T cells
NF κ B	Nuclear factor kappa B
NLS	Nuclear localization signal

List of abbreviations

NES	Nuclear export sequence
NPC	Nuclear pore complex
nPTK	Non-receptor protein tyrosine kinase
PKD1	Phosphoinositide dependent protein kinase 1
PH	Pleckstrin homology
PHLPP	PH domain and leucine-rich repeat protein phosphatases
PI3K	Phosphatidylinositol-3 kinase
PIN-1	Peptidyl-prolyl cis/trans isomerase-1
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PKB/AKT	Protein kinase B (AKT)
PKC	Protein kinase C
PLC γ	Phospholipase-C gamma
PRAS40	Proline-rich AKT substrate of 40 kDa
PTEN	Phosphatase and tensin homolog deleted on chromosomes 10
PTKs	Protein tyrosine kinases
RAS	Rat sarcoma
RBM25	RNA-binding motif protein 25
RLK	Resting lymphocyte kinase
SDS	Sodium dodecyl sulphate
SH1/KD	SRC homology 1/ C-terminal kinase/catalytic domain
SH2	SRC homology 2
SH3	SRC homology 3
SHP-1	SH2 containing phosphatase 1
SLP-65	SH2 domain containing leukocyte protein of 65 kDa
SYK	Spleen tyrosine kinase
TH	TEC homology
T _H	T _{Helper} cells
TXK	T and X cell expressed kinase
Xid	X-linked immunodeficiency
XLA	X-linked agamma-globulinemia
ZAP-70	z-chain associated protein tyrosine kinase of 70 kDa

1 INTRODUCTION

1.1 HEMATOPOIESIS

Hematopoiesis is the process of generation of all blood cell lineages originated from hematopoietic stem cells (HSCs), which resides in the Bone Marrow (BM) [1]. HSCs are capable of self-renewing and have the ability to give rise to the different blood cell types [2]. Various signaling molecules and transcription factors have been recognized to modulate the response of the downstream target genes required for hematopoietic lineage development [3]. The first progenitors to be generated from HSCs are the so-called multi-lineage progenitors (MLPs) that can differentiate into common lymphoid progenitor (CLP) or common myeloid progenitors (CMP) to produce all blood cells. The latter are Erythropoiesis (the formation of red blood cells), Leucopoiesis (the formation of white blood cells) and Thrombopoiesis (the formation of platelets) [2, 4]. Blood cells normally consist of red blood cells, platelets and white blood cells, which include granulocytes (neutrophils, eosinophils and basophils), and non-granulocytes (monocytes as well as T and B lymphocytes). Several human diseases affecting the hematopoietic system, such as leukemias and hemoglobinopathies are a serious public health problem [5].

The function of blood cells varies according to the cell types. For example, red blood cells transport oxygen to all tissues, while platelets take part in blood coagulation during wound healing. A major function of white blood cells is to provide protection against pathogens. The main white blood cells making up the immune system include Natural killer cells (NK cells), Dendritic cells (DCs), Neutrophils, Eosinophils, Basophiles, Macrophages, T cells and B cells and their secreted effectors [6, 7].

1.2 B LYMPHOCYTES (B CELLS)

B lymphocytes or B cells start maturation in the BM. During B cell development, in the Pro-B cell type, where the first gene rearrangement takes place. The Pro-B cells then developed to Pre-B cells, which express the μ chain on their surface. At this stage, B cells start to express surrogate light chains ($\lambda 5$ and VpreB) genes [8]. Next, B cells start to express functional Ig molecules on their surface and are transformed into immature B cells, which circulate in the periphery to become mature B cells. Thereafter, they distribute in the periphery for a short period and die unless they encounter antigen. B cell activation and IgD down regulation occurs following antigen binding in the lymphoid organs [9]. B cells are important members of the adaptive immunity and are responsible for the production of plasma cells and secretion of antibodies (immunoglobulins) against pathogenic microbes. In addition, B lymphocytes are involved in diverse immunological functions and are, in principle, considered as positive regulators

of the immune response. Generally, the role of B cells is to recognize extracellular pathogens that are internalized and subsequently processed to peptides for antigen presentation through MHC-II (major histocompatibility complex class II) molecules. By carrying out this elaborate task, B lymphocytes are able to provide supportive signals to specific $CD4^+$ T_{Helper} cells (T_H) [10]. Association of B cells and T_H cells facilitates the secretion of antibodies against pathogens, which are eliminated and therefore it is called T cell dependent (TD) B cell response. B cells can also stimulate immune response against pathogens without the help of T-cells, which is termed T cell independent (TI) response [11-13]. In addition to antibody (Ab) generation and antigen (Ag) presentation, B cells can also secrete different cytokines that influence the immune response [14, 15].

The IgM^+CD10^+ transitional cells are a subset of B lymphocytes that leave the BM and undergo several developmental phases as they migrate into the peripheral lymphoid tissues [16]. B cell expansion in the periphery is primarily dependent on the Ag-recognition process occurring in the secondary lymphoid organs, which serve as a microenvironment suitable for this event. In contrast, B cells that fail in Ag-recognition undergo apoptosis. Furthermore, B cells can proliferate and differentiate into memory cells as well as antibody (Ab)-secreting plasma cells (PCs) upon Ag encounter, cytokine signals and T cell interaction [17-19]. Additionally, B cell expansion, somatic hypermutation (SHM) and isotype-switching occur in the germinal center (GC) [20]. In the absence of antigenic stimulation, memory B cells can survive for a long period of time [21]. They can also enter the lymphoid organs upon antigenic stimulation and circulate in the periphery to participate in the secondary antibody response triggered by contact with T_H cells [22]. Two distinct groups of B lymphocytes exist; B1 and B2 cells. B1 cells generate normal Abs and mainly localize in the peritoneal cavity and gut-associated lymphoid tissues. B1 cells normally originate from the fetal-liver and have the capacity of self-renewing. B1 cells are also subdivided into two subsets based on the CD5 expression: B-1a cells ($CD5^+$) and B-1b ($CD5^-$) [23, 24]. In contrast to B1 cells, mature B2 cells are usually derived from the BM. These cells are further subdivided into follicular (FOL) B cells, which are localized in the secondary lymphoid tissues, and marginal zone (MZ) B cells confined to the MZ of the spleen [25].

1.3 B CELL MALIGNANCIES

B cell malignancies generally occur at all phases of B cell development. Any increase in the growth of lymphoid progenitor cells is known as acute lymphoblastic leukemia (ALL). ALL is one of the most common cancers in children, with 80-90% survival rate for children and 50% survival rate for adults [26]. Expansion of B-lineage cells comprises around 80% of ALL cases. The B-ALL type is characterized by CD10 expression with no cytoplasmic Immunoglobulins (Igs). B-ALL is classified into two types; pre-B-ALL (CD10 positive with cytoplasmic Igs) and pro-B-ALL (with no CD10 expression and no cytoplasmic Igs). Many chromosomal translocations have been found in leukemic B cells and are implicated in different malignancies. TEL-AML1 t(12;21) is a common translocation in children [27]. On the other hand, one of the most common translocations in adults is Breakpoint cluster region-Abelson murine leukemia viral oncogene homolog, [BCR-ABL t(9;22)] with poor treatment outcomes [27, 28]. Gleevec (Imatinib) is used as treatment for ALL patients with BCR-ABL translocations, however, there is a high risk of relapse due to drug resistance as a result of mutations in the ABL kinase domain. Therefore, new lines of tyrosine kinase inhibitors are being developed to treat these patients [29]. E2A-PBX1 and FLT3-ITD are other examples of common translocation in ALL as well as other malignancies have been shown that involve mixed-lineage leukemia gene (MLL) or MYC genes. Patients with these translocations are severely affected because of altered cell growth, increased cell survival and inhibited lymphocyte differentiation. A large percentage of ALL tumors lack translocations and are characterized by hyperdiploidy, with more than 50 chromosomes. Loss-of function mutations in genes involved in B cell development, such as Pax5, Ikzf1 (Ikaros), and Ebf1 are present in nearly 40% of ALL patients [30].

Expansion of malignant B cells, by chromosomal translocations, or mutations, has been linked to the deregulated activation of many protein tyrosine kinases and adaptor molecules [31]. For example, B cell linker protein/SH2 domain containing leukocyte protein of 65 KDa (BLNK/SLP-65) functions as a tumor suppressor in human pre-B cells; therefore, defective expression of BLNK/SLP-65 has been documented in nearly 50% of childhood pre-B ALL [32, 33]. Furthermore, it has been reported that the tumor frequency of pre-B-cell lymphomas considerably increases when expression of both BLNK and Bruton's tyrosine kinase (BTK) is compromised, indicating that BTK together with BLNK is important for suppressing leukemia [34]. Moreover, 16 pediatric patients with BLNK deficiency have been identified in a total of 34 patients with pre-B ALL [32]. Thus, the development of ALL seems to frequently occur as a result of the defect in some of the genes that play important roles in B cell development. Another common B cell malignancy is B cell chronic lymphocytic leukemia (B-CLL), which is the most common form of B cell leukemia in the

Western world [35]. B-CLL refers to the aberrant expression of CD5 on the accumulated monoclonal population of mature B cells [36, 37]. The most recurrent chromosomal aberration in CLL is deletion of chromosome 13q14. This region includes transcripts encoding miR15 and miR16, which are negative regulators of the anti-apoptotic gene B cell lymphoma 2 (*BCL2*) [38]. Another type of B-CLL with poor prognosis is caused by chromosome 17p deletion, which harbors the gene encoding for the tumor suppressor p53 protein [39, 40].

1.4 B CELL RECEPTOR (BCR) SIGNALING

Depending on the maturation stage of B cells, BCR-mediated intracellular signaling is important for B cell development, proliferation and survival. For example, BCR signals stimulate the proliferation and survival of mature B cells. In contrast, these same signals, enhance apoptosis or inactivation via anergy or receptor editing in immature B cells [41]. Functional BCR signaling complex comprises of two transmembrane immunoglobulin heavy chains and two light chains. BCR is regularly referred to as surface IgM with a very short cytoplasmic domain. Therefore, BCR is fixed inside the membrane and connected to transmembrane heterodimer components, Ig α (CD79 α) and Ig β (CD79 β), which are necessary for transducing the signals [42, 43]. The cytoplasmic terminal portion of Ig α and Ig β contain immunoreceptor tyrosine-based activation motif (ITAM), which is essential for protein binding and transduction of downstream signaling [44, 45]. BCR aggregation occurs in lipid rafts upon antigen binding, which enables SRC-family kinases, LYN, FYN, LCK or BLK to phosphorylate the tyrosines in the ITAM [46, 47]. Moreover, phospho-tyrosines in vascular endothelial growth factor receptor 2 (VEGFR2) are important for the recruitment of SRC family kinases in vascular endothelial cells [48] and BMX/ETK in endothelial cells [49, 50]. Phosphorylated tyrosine residues in the ITAM recruit kinases such as spleen tyrosine kinase (SYK) leading to their activation and subsequent formation of the signalosome, involving SYK, BLNK, BTK, PI3K, PLC γ 2, VAV, PKC β , CARMA1, as well as other proteins that propagate activation signals to downstream secondary effectors [44, 45, 51-53].

Several positive and negative co-receptors of the BCR are present on the B cell surface and are important for the regulation of BCR signaling. CD19 and CD45 enhance BCR signaling [54, 55], whereas CD22, Fc γ RIIb and PIR-B attenuate BCR signaling. LYN can phosphorylate CD19, which creates docking site for the SRC homology 2 (SH2) domain of phosphatidylinositol-3-kinase (PI3K) leading to generation of phosphatidylinositol 3,4,5-triphosphate (PIP3) from phosphatidylinositol 4,5-bisphosphate (PIP2) (Figure 1). The plasma membrane-localized phosphatase CD45 can stimulate BCR signaling through dephosphorylation of an inhibitory tyrosine in the tail of LYN, which is

phosphorylated by the C-terminal SRC kinase, CSK [55]. In contrast to the stimulatory effect of LYN on BCR signaling, LYN phosphorylates ITIMs (immunoreceptor tyrosine-based inhibition motif) of the CD22 and FcγRIIB inhibitory receptors through recruitment of SH2 containing phosphatase (SHP-1) and SH2 containing inositol phosphatase (SHIP-1) as well as preventing PI3K binding to CD19 [54, 56, 57].

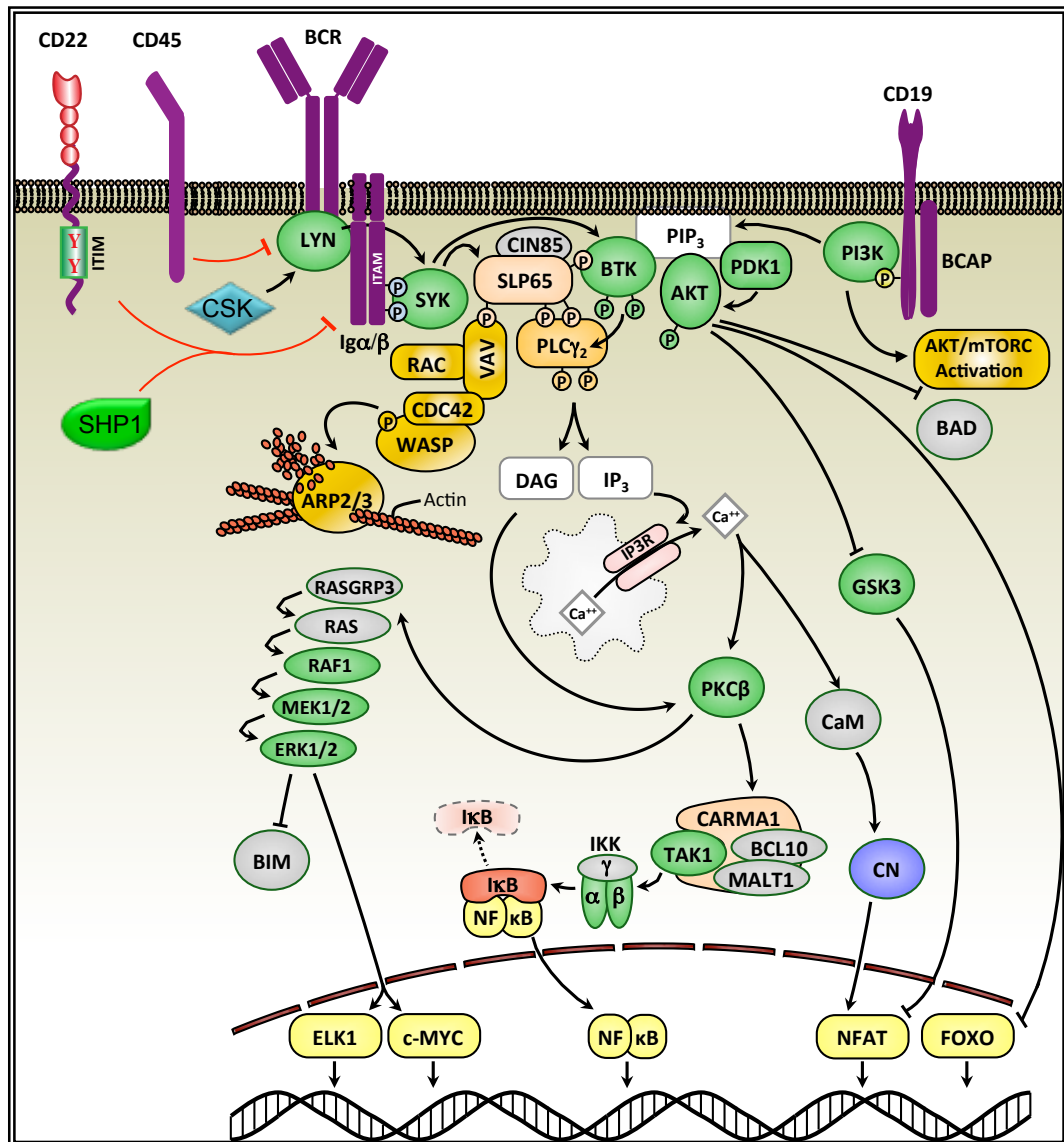


Figure 1. B cell receptor signaling cascade. BCR transduction is elicited upon antigen binding. The balance of the initiation, amplitude and length of BCR stimulation is influenced by different molecules, such as kinases (like LYN, SYK, PI3K), adaptors (such as BLNK/SLP65, BCAP, CARD11), and the co-regulators (for example CD22, CD19, CD45). Modified from [58].

The B cell cytoplasmic adaptor protein (BCAP) can recruit PI3K to the membrane upon BCR oligomerization to facilitate generation of PIP3 from PIP2. PIP3 acts as a docking site at the plasma membrane for pleckstrin

homology (PH) domain containing proteins [59]. Several cytoplasmic tyrosine kinases and molecules are recruited to the plasma membrane through their PH domain, following PIP3 production. After BCR stimulation, activated phospholipase-C gamma2 (PLC γ 2) generates the secondary messengers; inositol triphosphate (IP3) and diacylglycerol (DAG) [60]. Protein kinase C (PKC) and calcium mobilization are then activated by DAG and IP3 respectively [61], leading to the activation of nuclear factor kappa B (NF κ B), nuclear factor of activated T cells (NFAT) transcription factor families and JNK [62, 63].

1.5 PROTEIN TYROSINE KINASES

Protein tyrosine kinases (PTKs) are important molecules in multicellular organisms and are involved in various signal transduction pathways. The PTKs are divided into a receptor protein tyrosine kinases (rPTK) group and a cytoplasmic, non-receptor protein tyrosine kinase (nPTK) group [64, 65]. The nPTK group is also subdivided into several families, including SRC, which is the largest family in this group of kinases and TEC family kinases (TFKs), the second largest family [66].

1.5.1 TEC Family Kinase (TEC)

The TEC family kinases are cytoplasmic non-receptor tyrosine kinases found primarily, but not exclusively, in hematopoietic lineages, where they are differentially expressed. Members of this family are: Tyrosine kinase expressed in hepatocellular carcinoma (TEC), BTK, IL2-inducible T cell kinase (ITK/EMT/TSK), BM tyrosine kinase gene in chromosome X (BMX/ETK) and Resting lymphocyte kinase RLK/TXK [67]. These kinases, which have been extensively studied, have broadened our understanding regarding lymphocyte development and signaling in both cell lines and animal models. They are often involved in a variety of cellular processes, including calcium (Ca²⁺) mobilization and activation of PLC γ 2, actin reorganization, adhesion, migration (motility) and survival/apoptosis [68]. The structures of TEC family members are 50-60% conserved and have a similar domain structure. The TEC family members are multi-domain proteins consisting of N-terminal pleckstrin homology (PH) domain, SRC homology domains 3, and 2 (SH3, SH2), and a C-terminal kinase/catalytic domain (SH1/KD). TEC family kinases contain an amino-terminal PH domain, which is common in several intracellular signaling molecules and is able to bind PIP3 for mediating protein-protein interactions [69].

1.5.2 Bruton's Tyrosine Kinase (BTK)

BTK is a cytoplasmic tyrosine kinase belonging to the TEC family of kinases. BTK activity is specifically critical for B-lymphocyte development and

signal transduction through various receptors, such as FcεRI, interleukin 3 (IL-3), interleukin 6 (IL-6) and G-protein coupled receptors (GPCR) [66, 70-72]. Thus, BTK is a central player in pre-B and B-cell signaling initiated by the BCR and the pre-BCR, controlling development, proliferation and differentiation of B-cell lineages [73]. A point mutation affecting a conserved Arginine (Arg) in the PH domain of BTK causes immunodeficiency due to lack of B lymphocytes and Abs in humans, a condition called X-linked Agammaglobulinemia (XLA). In mice, a similar mutation leads to X-linked immunodeficiency (*Xid*), a far less severe phenotype [74, 75].

In 1952, O. C. Bruton discovered a recessive genetic disorder characterized by the absence of immunoglobulins, named XLA in patients with recurrent bacterial and enteroviral infections [76]. The symptoms appear at a very early age with a lack of humoral immunity due to a distinct decline in serum Igs of all classes [77, 78]. XLA is initiated after maternal Igs are catabolized and absence of L-chain re-arrangements leads to negligible levels of new B-lymphocytes and plasma cells being formed. In XLA patients, B-lymphocyte development is defective with a partial block after pro-B-cell and a complete blockage after pre-B-cell phase, leading to absence of B-lymphocytes in humans (Figure 2). In contrast, there is only a partial block in *Xid* mice [79, 80]. It has been shown in 1993 that the molecular basis of XLA defect is due to mutations in a tyrosine kinase, termed BTK [70, 71, 81]. Using different approaches, the defective *BTK* gene in XLA was mapped to the long arm of the X chromosome (Xq 21, 3-22 region [82-85].

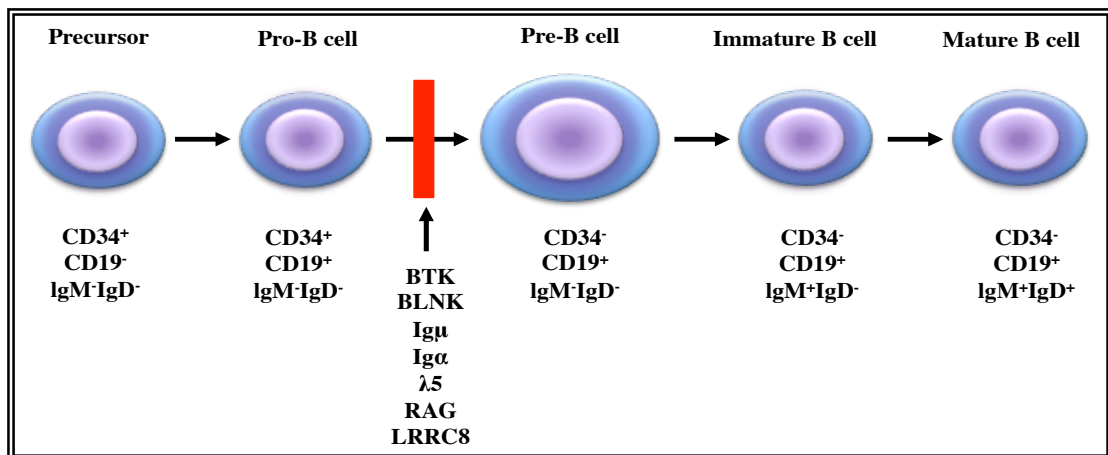


Figure 2. Early stages of B-cell differentiation can be identified by the genes, the cell surface markers CD34, CD19, and surface immunoglobulin (sIg).

In addition to an N-terminal PH domain, the BTK protein contains SH3, SH2 and SH1/KD and an area of 60-80 amino acids among the PH and SH3 domains named the TEC homology domain (TH). The PH domain of BTK associates with numerous proteins, such as PKC, βγ-complexes of heterotrimeric G-protein, PIP3, PIN-1, transcription factor (TFII-I), VAV, FAS, F-actin and focal adhesion kinases (FAK) [86-90] (Figure 3). TH domains contain proline-

rich sequences and have been suggested to be involved in the auto-regulation of BTK.

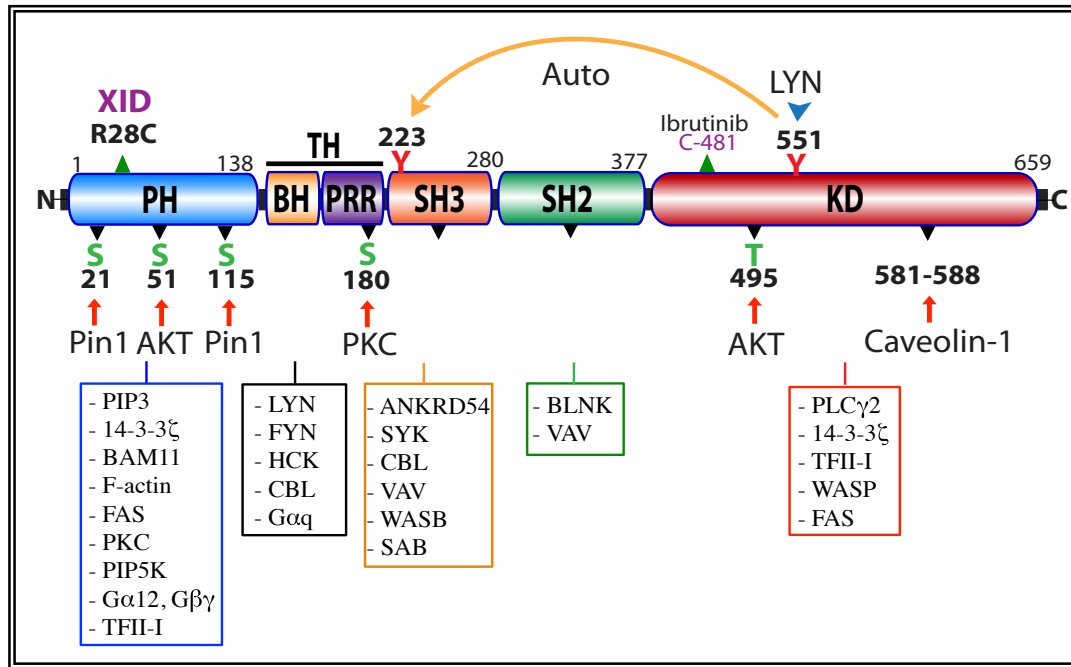


Figure 3. Schematic representation showing the BTK structure. The phospho-tyrosines pY223 and pY551 are in red and the regulatory serine/threonine phosphorylation sites are in green. Arrows in each domain indicate the interacting partners and/or regulating proteins. The R28C mutation found in XID mice causes classical XLA in humans.

Following BCR stimulation with antigen, tyrosine residues in the cytoplasmic tails of Igα/Igβ heterodimers are phosphorylated within the ITAM. Members of SRC family kinases carry out the phosphorylation of these residues creating docking sites for SYK tyrosine kinases. PI3K is also activated by BCR antigen engagement and results in an increase in the PIP3 in the plasma membrane, leading to the plasma membrane translocation of many signaling proteins including Tec family kinases through their PH domain. BTK signaling is believed to predominantly occur in the plasma membrane together with other signaling components assembly that leads in the formation of BTK-signalosome [72, 91-94]. The membrane-localized BTK is active following transient phosphorylation of two of its highly conserved tyrosine residues, tyrosine Y551 and tyrosine Y223. Tyrosine Y551 in the activation loop of the kinase domain is trans-phosphorylated by the SRC family tyrosine kinase LYN. Phosphorylation at Y551 induces BTK to undergo a conformational change leading to auto-phosphorylation at tyrosine residue Y223 within the SH3 domain resulting in increased kinase activity [87]. Phosphorylated BTK brings PLCγ2 and BLNK in close proximity with SYK, leading to tyrosine phosphorylation of PLCγ2. Phosphorylated and activated PLC-γ2 hydrolyzes PIP2 into IP3 and DAG, followed by calcium mobilization and PKC activation [46].

Since BTK does not have a negative auto-regulatory mechanism to modulate its own activity, it seems to entirely depend on the interacting signaling partners to regulate kinase activity. Interestingly, a number of well-known BTK-interacting proteins were shown to function as a negative feedback regulator to fine-tune BCR signaling through BTK, such as PKC β , Caveolin-1, Peptidylpropyl *cis/trans* isomerase (PIN-1) and AKT. PKC β was found to down regulate BTK activity via direct phosphorylation at Ser-180, which subsequently reduces membrane recruitment, trans-phosphorylation and retains BTK in the cytoplasm [95]. It has been shown that BTK interaction with caveolin-1 leads to down regulation of the BTK kinase activity [96]. The two identified serine residues, Ser-21 and Ser-115, of BTK phosphorylated by Pin1, are prerequisites for negative regulation of BTK. Phosphorylation of serine 21 leads to Pin1 binding and guiding BTK during mitosis, whereas serine115 phosphorylation leads to Pin1 interaction with BTK in resting cells [86]. Recently, we have shown that AKT-induced phosphorylation of BTK at residues Ser-51 and Thr-495 is required for the 14-3-3 ζ interaction and subsequent degradation. In contrast, PKC θ activates BTK, whereas BTK down regulation results in the induction of the PKC θ activity [97].

Activation of TEC family kinases is mainly dependent on the synergistic action of PI3K and SRC family kinases activity [98], together with JAK/SYK family members [99]. The erythropoietin receptor (EPOR) is expressed on B-lymphocytes and is modulated when treating with recombinant human erythropoietin (EPO) [100]. EPO induces downstream signaling by activating proteins such as PLC γ and Signal Transducer and Activator of Transcription 5 (STAT5). On the other hand, BTK is phosphorylated by Janus kinase 2 (JAK2) in response to EPO [101].

1.5.3 Spleen Tyrosine Kinase (SYK)

SYK is a member of the nPTK family together with zeta-chain associated protein kinase 70 (ZAP70) [102]. SYK is an essential player for signal transduction initiation in a variety of cell types. The activity of SYK is critical in the development of B-cells progenitors and plays a key role in the uncontrolled growth of tumor cells, in particular, those of B cell origin [103, 104]. This kinase functions downstream of both antigen receptors (BCRs) and Fc receptors (FcRs) in various cells and transduce signals leading to calcium mobilization, altered gene expression, differentiation, phagocytosis, cell proliferation, survival and cytokine production [105, 106]. Moreover, SYK is essential for platelet function, particularly in the initiation of some of the integrin, C-type lectin CLEC-2, and GPVI receptors [107]. SYK is abundantly expressed in a wide variety of cells including all hematopoietic lineage cells as well as non-hematopoietic cells such as leukocytes, macrophages, mast cells, platelets, erythrocytes, hepatocytes, osteoclasts, fibroblasts, epithelial cells, neuronal and vascular endothelial cells

[106, 108, 109]. SYK associates with transmembrane proteins containing ITAM upon phosphorylation of the two-tyrosine residues [106]. Recruitment and activation of SYK or ZAP70, (the second SYK family protein) in B cell or T cells respectively, results in activation of different cellular processes [106].

In addition to the above mentioned functions, SYK activation, downstream of Dectin-1 in response to microbes to mediate inflammation and immunity, results in the production of ROS, activation of MAPK including ERK and activation of NFAT by CARD9–BCL-10–MALT-1 and more recently, CARD9–H-RAS–RAS–GRF1 signaling complex [110-112]. Moreover, activated SYK leads to activation of NFκB via CARD9 as antifungal immunity [113]. Therefore, DCs and macrophages with SYK deficiency display impaired IL-2, IL-10 and ROS production in response to fungal stimulation [114-116]. SYK also has been linked to the regulation of IL-1β production in response to fungal infection [114].

SYK comprises of two-tandem SH2 domains, which are linked by an amino acid stretch called interdomain A, and a C-terminal tyrosine kinase domain joined with C-terminal of the second SH2 domain via interdomain B linker region [117, 118] (Figure 4). Two isoforms of SYK have been described as a result of alternative splicing, SYK(L) and SYK(S)/SYKB. SYK(S) has identical structure compared to SYK(L), but lacks a 23 amino acid stretch in its interdomain B, which has been reported to confer nuclear localization [119, 120]. SYK(L) is predominantly expressed in the B-cell lineage, whereas the shorter isoform SYK(S) is mainly expressed in the BM [121, 122]. The longer isoform, SYK(L) is found in both cytoplasm and nucleus, whereas the SYK(S) is confined to the cytoplasm [119] .

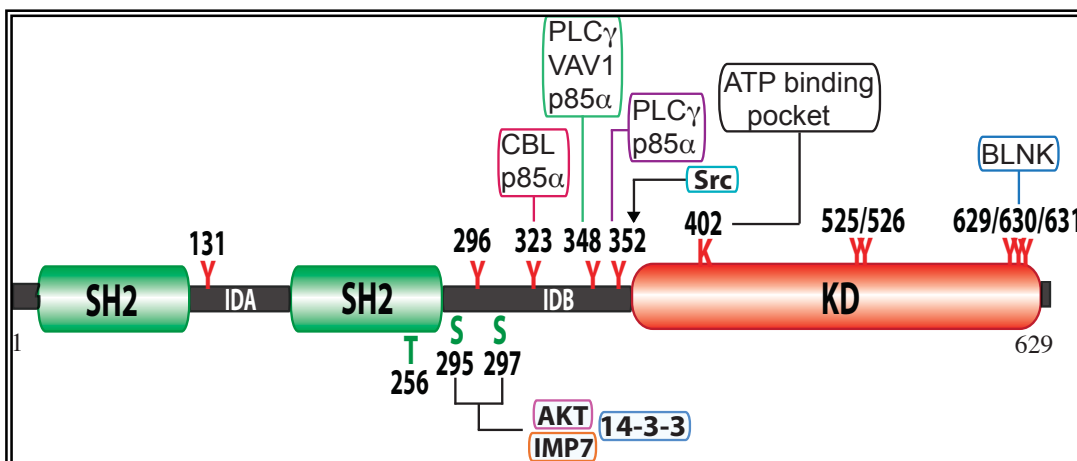


Figure 4. Schematic representation of SYK domain structure showing interacting protein partners and regulatory phosphorylation sites. Tyrosines are in red color and serine/threonine in green.

SYK activation loop tyrosine residues (Tyr525/526) phosphorylation by SRC family kinases is required for full activation of the kinase [123]. Moreover, autophosphorylation of the linker tyrosine residues of SYK maintains its activity in an ITAM independent fashion [124]. Thus, SYK is a proximal signal transducer element of the BCR. This, in turn, couples the BCR to the activation by recruiting various binding partners, such as, BLNK, PLC γ 2/PKC, PI3K/AKT, VAV, NF κ B and RAS/RAF/ERK [125-133]. In the absence of SYK, few if any signals are sent following BCR clustering [134]. SYK stabilizes its open conformation (catalytically active form) by employing both a phosphorylation and protein-protein interaction approach. Notably, SYK has the capability to establish its own positive feedback loop mechanism that can convey ITAM tyrosine phosphorylation as well as its kinase domain phosphorylation, independent of SRC family kinases [135]. Moreover, various negative regulators of SYK have also been demonstrated. For example, SHP-1 phosphatase keeps SYK in a dephosphorylated form [136]. Another possible mechanism for the negative regulation of SYK is by the E3 ubiquitin ligase, Casitas B-lineage lymphoma (CBL) [137]. Once the BCR is stimulated, CBL binds the phosphorylated Tyr323 in the interdomain B region of SYK, which facilitates ubiquitination as well as proteasomal degradation of SYK [137]. That said, it has been shown that Tyr130 phosphorylation in the interdomain A of SYK enhances detachment from ITAM, which induces SYK down regulation [138, 139].

To date, two different chromosomal translocation events involving SYK have been identified that give rise to chimeric oncogenes, TEL-SYK and ITK-SYK [140, 141]. TEL-SYK translocation has been detected in a single patient with myelodysplastic syndrome, while ITK-SYK translocation was recurrently identified in a subset of peripheral T cell lymphomas. Defects or elevation in the expression of SYK has been described in B cell lymphomas because of impairments in the differentiation of B-lineage cells [103, 142-145]. Also, SYK deficiency has been reported as tumor promoting in breast cancer and melanoma [146, 147], whereas, loss of ZAP70 expression results in reduced T lymphocyte mediated immunity [148]. Because of the important role of SYK in lymphocyte differentiation and proliferation, different hematological malignancies and cell transformation have been linked to abnormality of SYK such as pre-B ALL and B-CLL [106, 149]. Therefore, developing novel compounds that block SYK activity or suppress gene expression of SYK have been examined and used in clinical trials for treatment of such disorders [149-152].

1.6 SERINE AND THREONINE KINASE FAMILY

1.6.1 AKT/PKB structure and signaling

The protein kinase AKT, also known as protein kinase B (PKB), is a serine/threonine kinase that functions downstream of the PI3K signaling pathway. AKT signaling plays an important role in the regulation of a plethora of cellular signaling events modulating cell growth, proliferation, differentiation, survival, glucose uptake, metabolism and angiogenesis [153, 154]. Three isoforms of AKT proteins AKT1/PKB α , AKT2/PKB β and AKT3/PKB γ have been identified in humans. The genes encoding these proteins are located on separate chromosomes. AKT proteins are members of the AGC kinase family with distinct physiological functions, expression and characteristics [155-157] (Figure 5). AKT1 is ubiquitously expressed in most tissues and is mainly involved in cell growth and survival, while AKT2 expression is limited to skeletal muscle, heart, kidney, pancreas and liver and is largely involved in insulin signaling and glucose homeostasis. On the other hand, AKT3 expression is restricted to the neural tissue and its function is critical for testis- and brain development [158-160]. Recently, the involvement of AKT3 in breast cancer aggressiveness has also been described [161]. AKT1 knockout (KO) mice show dwarfism phenotype in which a reduction in body and cell size occurs [162]. Deletion of AKT2 leads to insulin resistance and a diabetes mellitus-like syndrome [163]. Deficiency in AKT3 leads to decrease in brain size and disorganization of corpus callosum [164]. Importantly, double KO of both AKT1 and AKT2 leads to neonatal lethality [165]. The different AKT isoforms have a similar structure consisting of PH domain in the N-terminal and α -helical linker domain followed by a kinase domain and a hydrophobic regulatory motif near the C-terminus [166, 167].

AKT is recruited and translocated to the membrane via PH domain binding to PIP3, where it is activated by PI3K [168, 169]. Following recruitment of AKT from the cytosol to the inner leaflet of the plasma membrane and binding to PIP3, the AKT conformation is altered, subsequently exposing threonine 308 (Thr308) in the activation loop of the kinase domain (residue numbers correspond to AKT1) for phosphorylation by the serine/ threonine phosphoinositide dependent protein kinase 1 (PDK1), which is also recruited to the membrane via its PH domain upon PI3K activation [61, 157, 170, 171]. The phosphorylation of AKT at Thr308 in the kinase domain is important for the partial AKT activity, which is sufficient to phosphorylate and inactivate proline-rich AKT substrate of 40 kDa (PRAS40) and tuberous sclerosis protein 2 (TSC2), which subsequently can directly or indirectly activate mTORC1 [172]. Activated mTORC1 further phosphorylates its key substrates, eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), and ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (S6K-1), which promote protein synthesis and

cell proliferation [173]. The mTORC1 structure components are composed of a regulatory-associated protein of mTOR (Raptor), PRAS40, mammalian TORC subunit LST8 (mLST8)/G-protein β -subunit like protein (G β L), and DEP domain containing mTOR-interacting protein (Deptor) [174].

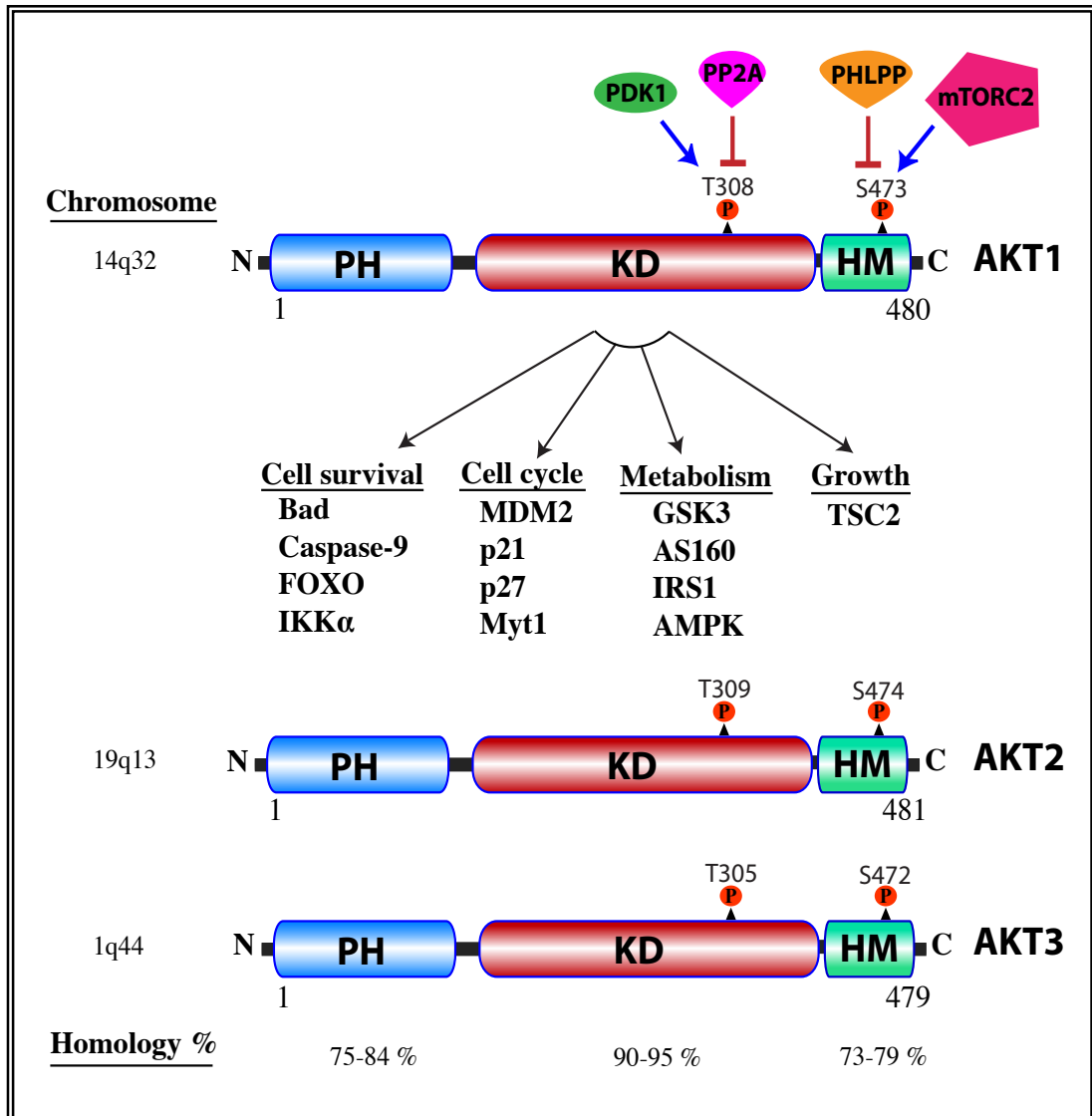


Figure 5. Domain structure of AKT isoforms showing the phosphorylation sites and regulatory partners.

Recently, it has been shown that mTOR complex 2 (mTORC2), especially Rictor, can further phosphorylate AKT at Ser473 in the carboxyl terminal hydrophobic domain. Full activation of AKT leads to relocation to distinct compartment to further phosphorylate additional substrates containing RXXXS/T motif in the target proteins [169, 170, 175-178]. To date, numerous AKT interacting proteins are shown to harbor the RXXRXS/T motif. Phosphorylation of this motif creates a docking site for 14-3-3 proteins [179, 180]. At this point, AKT/PKB full activation can mediate various functions including angiogenesis, metabolism, growth, proliferation and survival/apoptosis [181]. The mTORC2 comprises of several components, involving mTOR, Rictor

(Rapamycin insensitive-companion of mTOR), mLST8, Deptor, mSin1 (mammalian stress-activated protein kinase interacting protein), and Protor (protein observed with Rictor-1) [182, 183]. AKT has a transitional role between two complexes, mTORC1 and mTORC2. mTORC1 functions downstream of AKT, while mTORC2 is known to be an upstream regulator of AKT kinase activation [182]. Thus, AKT plays a central role in the cross talk between many cellular signaling processes and also acts as a proto-oncogene, which can contribute to the development or progression of various human cancer forms [184, 185]. Notably, PI3K/AKT pathway is also active downstream of platelet-derived growth factor receptor B (PDGFRB), fibroblast growth factor receptor (FGFR) and VEGFR2 [186, 187]. Tyrosine residues in FGFR create a binding site for PI3K/AKT as well as PLC/PKC and RAS/MAPK. Thus, growth induction of various tumors have been observed due to increased and dysregulated PDGFRB and FGFR levels [186]. Additionally, AKT activity is inhibited by mutating domain B of Neuropilins 2 (NRP2), which is a co-receptor for VEGF through blocking of VEGF binding to NRP2 [188].

Additional proteins are known to be responsible for dephosphorylating AKT. Various phosphatases have been shown to dephosphorylate AKT and negatively regulate AKT activity. Recently, the PH domain and leucine-rich repeat protein phosphatases PHLPP1 and PHLPP2 [189] have been reported to dephosphorylate S473 of AKT2 and AKT1, respectively [190]. Phosphatase, PP2A inactivates AKT by direct dephosphorylation of the Thr308 residue, and Phosphatase and tensin homolog deleted from chromosome 10 (PTEN) converts PIP3 to PIP2 by dephosphorylating the 30-position of the inositol ring in PIP3, which results in PIP2 production (Figure 6). Thus, PHLPP1/2, PP2A and PTEN together participate in the termination of AKT signaling [191-195]. Notably, PTEN mutations or loss of its expression constitutively activates AKT signaling, subsequently leading to increased growth and prevent apoptosis. Furthermore, deficiency of both PHLPP and PTEN is highly correlated with prostate cancer [196]. Moreover, mTORC2 activity can be regulated by Sin1 phosphorylation at Ser260, which inhibits lysosomal degradation of Sin1 and subsequently increases the integrity of mTORC2 [197]. Another way of mTORC2 regulation is through Glycogen synthase kinase-3 (GSK-3 β) induced Rictor phosphorylation at Ser1235 leading to mTORC2 inactivation, which affects the capability of substrate binding [198]. Deregulation of the PI3K/AKT pathway and increased levels of phosphorylated AKT has been reported in numerous cancers, which is concurrent with severe phenotype and poor prognosis. Therefore, a balance between the activity of PDK1/AKT/mTORC2 and PHLPP/PP2A/PTEN is required for controlling the kinase activity of AKT.

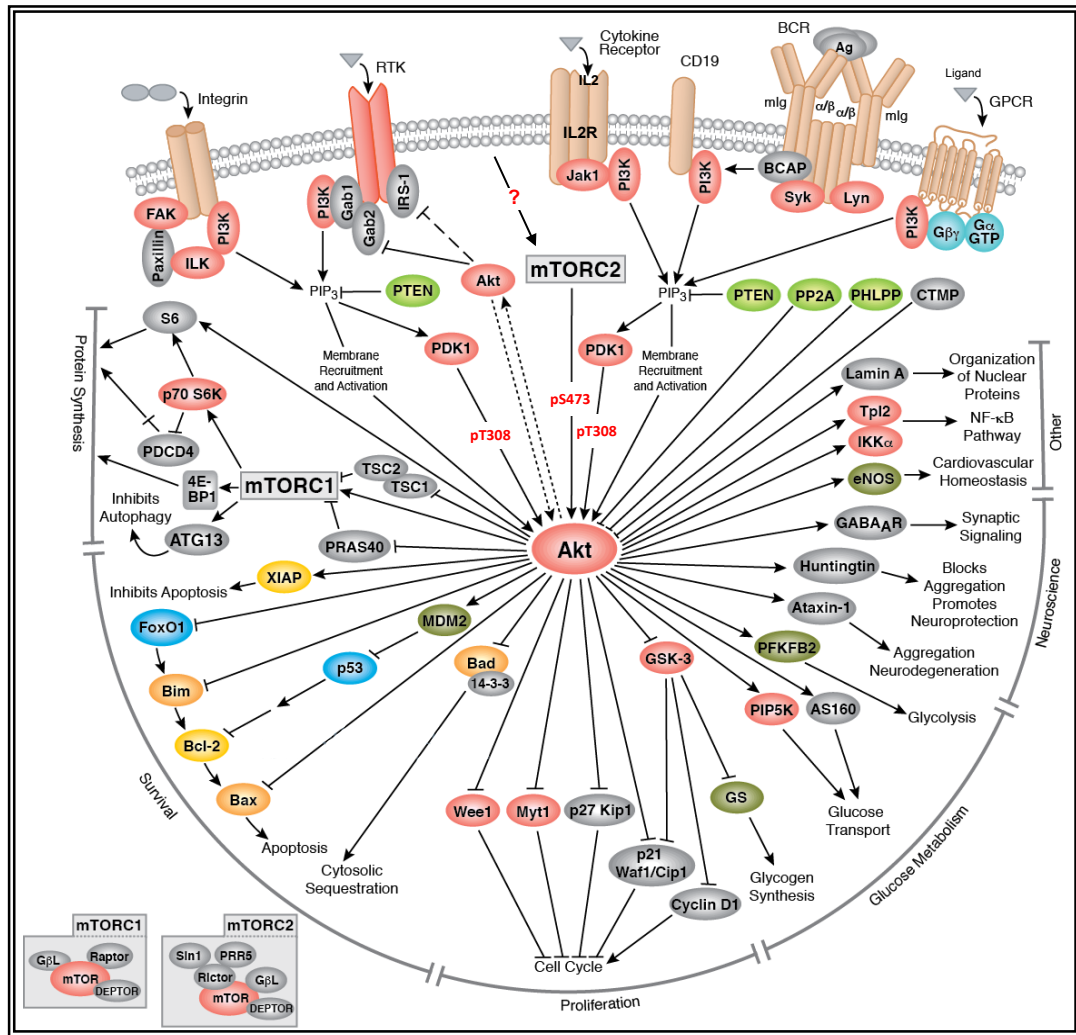


Figure 6. Schematic representation of AKT activation and downstream signaling pathways. "Illustration reproduced by courtesy of Cell Signaling Technology, Inc. (www.cellsignal.com) with slight modification".

1.7 ADAPTOR MOLECULES

1.7.1 14-3-3 family proteins

The 14-3-3 family proteins are highly acidic proteins expressed in all eukaryotic cells [199, 200]. 14-3-3 proteins regulate various cellular processes, in particular, apoptosis and cell-cycle checkpoints [201]. In mammals, seven isoforms constitute this family denoted as 14-3-3 β , γ , ϵ , ζ , η , θ and σ [202]. It has been reported that the 14-3-3 alpha isoform is the phosphorylated form of 14-3-3 beta, while 14-3-3 delta isoform is the phosphorylated form of 14-3-3 zeta [203]. In cells, the overwhelming majority of 14-3-3 proteins exist as both homodimers and heterodimers. However, the sigma isoform, which only forms homodimers, is the exception [204-206]. 14-3-3 proteins generate dimers that creates docking sites for target molecules, functioning as scaffold protein [199]. In addition to serine/threonine phosphorylation, association of 14-3-3 also requires the full 14-3-3 consensus-motif for complete binding [207]. 14-3-3

proteins also act as regulators of many biological as well as cellular functions involving cell cycle progression, metabolism, apoptosis, cytoskeleton regulation and cytoplasmic sequestration. Cytoplasmic sequestration further facilitates targeted protein stimulation/prevention of enzymatic activity, degradation, and enabling of protein modification. Therefore, lack of expression and/or function of 14-3-3 proteins could contribute to a disarray of cellular activities [208-210]. 14-3-3 proteins bind to three different consensus-binding motifs; RSXpTXP, RXY/FXpTXP and AKT phosphorylation site (RXRXXpS/T), where pT/S denotes phospho-serine/threonine [211]. Binding of 14-3-3 to the target protein is dependent on the phosphorylation of serine or threonine, which permits the conditional interaction of 14-3-3 with protein partners harboring RXRXXpS/T motif [211-213]. Moreover, 14-3-3 monomer consists of an amphipathic ligand-binding groove permitting each 14-3-3 to bind to two different residues of the same protein or two different proteins simultaneously.

The modes of action of 14-3-3 proteins can be generally classified into the following categories: (1) direct conformational change of the target protein; (2) preventing the target protein from dephosphorylation (3) as adaptors (scaffolding molecules), mediate anchoring of proteins in close proximity of one another and (4) modulating subcellular localization of target proteins [201, 214, 215]. Nucleocytoplasmic trafficking of proteins is necessary in the regulation of various cellular functions. It has been shown that 14-3-3 ζ is present in the nucleus and can rapidly shuttle between cytoplasm and the nucleus [216]. 14-3-3 binding can increase the nuclear export or decrease nuclear import for the target protein [217]. 14-3-3 proteins have a nuclear export signal (NES) that can bind to the chromosome maintenance region 1 (Crm1), leading to export of 14-3-3 from the nucleus [218, 219]. The NES sequence in 14-3-3 proteins contains amino acids that are also involved in the target binding and thereby compete with the Crm1 [220]. The reason that 14-3-3s can be found in the nucleus is because the target protein occupies the NES sequence and this indicates that the NES signal can be masked for export [221, 222]. However, when the NES signal is uncovered, the 14-3-3 can be exported from the nucleus together with its target.

The involvement of 14-3-3 proteins in human cancers is just beginning to come to light. Two 14-3-3 family members, 14-3-3 ζ and 14-3-3 σ have been reported to be frequently correlated with tumor initiation and progression. 14-3-3 ζ is implicated in oncogenesis via its association with various cancer initiation and progression proteins (BAD, RAF, p85PI3K, FOXO, SNAIL, TGFbetaRI). In addition, overexpression of 14-3-3 ζ leads to the activation of PI3K-AKT signaling pathway thereby down regulating the tumor suppressor p53 [223, 224]. Expression of 14-3-3 ζ has been reported to be elevated in different human cancers, such as stomach cancer, hepatocellular carcinoma and breast cancer [225]. As mentioned above, an abnormal increase in the steady-state levels of

14-3-3 ζ is now considered as a marker for a variety of tumor types [226]. In contrast to 14-3-3 ζ , 14-3-3 σ functions as a tumor suppressor, and reduced expression of this protein has been observed in various tumors, such as lung carcinomas and breast cancers [227, 228]. The tumor suppressor function of this isoform has been attributed to its positive effect in regulating p53 and controlling a G2/M checkpoint following DNA damage [229]. In this regard, the development of small molecule inhibitors such as BV02, is needed to target 14-3-3 and exclusively prevent the association of 14-3-3 with partner proteins and is promising for anti-cancer therapies [230-232].

Notably, 14-3-3 proteins can also be regulated at the level of phosphorylation by different kinases. This regulatory mechanism, which is currently gaining momentum, suggests that phosphorylation of 14-3-3 at different sites (such as Ser58, Ser185 and Thr233) negatively affects association with a target proteins and disrupt dimerization of 14-3-3. Various kinases have been shown to phosphorylate 14-3-3 at particular sites including certain PKC isoforms, PKA, AKT, JNK, CKI and BCR kinases [233-238]. Moreover, K49 and R56/R60 are key residues in the binding groove of 14-3-3, which mediate binding of these proteins to phosphoserine/threonine sites of target proteins. Accordingly, mutation of these residues to alanine impairs target-binding ability [239, 240].

1.7.2 ANKRD54 (LIAR)

Ankyrin repeat domain 54, ANKRD54 (also called Lyn-interacting ankyrin repeat, LIAR) is a 300 amino acid scaffold protein corresponding to a 34 kDa polypeptide. ANKRD54, a cytoplasmic and - localized protein, is important for the assembly of multiple intracellular signaling molecules and functions as a scaffold that can regulate signal transduction complexes. The nucleo/cytoplasmic shuttling ability of ANKRD54 is due to the presence of a functional nuclear localization signal (NLS) and an NES. ANKRD54 (LIAR) is widely expressed in various tissues, such as pancreas, prostate, spleen, leukocytes, placenta and brain [241]. Furthermore, ANKRD54 has also been recognized as a highly expressed protein in ciliated cells [242].

ANKRD54 consists of 4 ankyrin repeats in the center of the protein and is enclosed by a bipartite NLS motif in the N-terminus and NES motifs in the C-terminus [241, 243] (Figure 7). The gene encoding human ANKRD54 maps to chromosome 22q13.1 and to chromosome 15 E1 in mouse [244]. Notably, chromosome 22q13.1 in humans has been linked to leukemia and other cancers. The structure of ANKRD54 displays a highly conserved sequence among mouse, rat and human [241]. The ankyrin repeats domains have been reported to exhibit specificity for protein-protein interaction [106, 245, 246] Ankyrin repeat domain sequences occur in approximately 6% of all eukaryotic proteins and

typically consist of 33 residues with two antiparallel-helices linked via a loop in the core and a pair of β -hairpin at both ends [246]. Moreover, the N-terminal region of ANKRD54 contains a potential ATP-binding P-loop (51 GLPGRS 56) in the mouse, but not in the human protein [243].

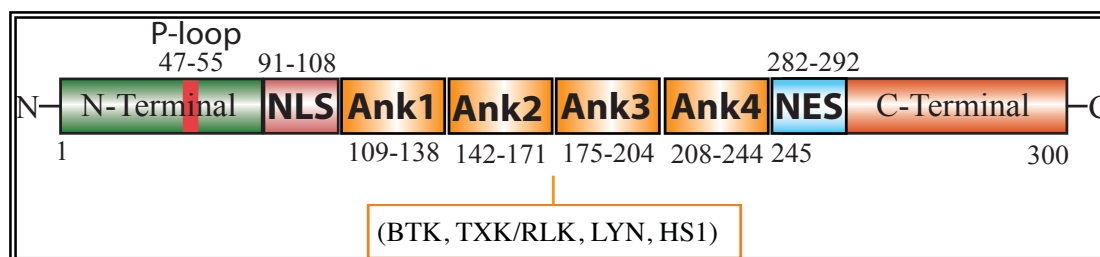


Figure 7. Domain structure of ANKRD54 (LIAR) and interacting protein partners.

Recently, ANKRD54, with its ankyrin repeats, has been shown to be an important partner for various proteins containing SH3 domain, such as ESE2L, LASP1, VAV1, Hip55, LYN, HS1 and BTK [241, 243]. Interestingly, the interaction of ANKRD54 with the partner proteins seems to be independent of the canonical proline-rich consensus binding motif in the SH3 domain [244]. Recently, we have demonstrated that ANKRD54 directly interacts with BTK and regulates its nucleo/cytoplasmic shuttling in an SH3-dependent manner [241]. Similarly, ANKRD54 also associates with Lyn through the SH3-domain by forming a multiprotein complex that influences erythropoietin-induced differentiation of erythrocytes [243]. Importantly, the association of ANKRD54 with LYN or BTK is independent of the phosphorylation status and kinase activity of these proteins. Moreover, at present, there is no evidence suggesting that LYN phosphorylates LIAR [241, 243]. The ankyrin repeats of I κ B interact with p65 subunit of NF κ B, which inhibits p65 translocation to the nucleus and thus abolishes the activation of the NF κ B signaling cascade [247, 248].

1.7.3 BLNK (SLP-65)

BLNK (also called SLP-65 or B cell adaptor containing an SH2 domain, BASH) is a cytoplasmic central adaptor protein in B cells without intrinsic catalytic function [249, 250]. BLNK, is considered as initial substrates for PTKs following BCR-activation [250]. In human B cells, in addition to the full-length protein, an alternative splice variant lacking exon 8 (corresponding to amino acids 760-828 in the proline-rich domain of BLNK) exists and is referred as BLNK-S [251]. Upon BCR engagement and ITAM phosphorylation, BLNK can be recruited to the plasma membrane and is phosphorylated by SYK kinase at different tyrosine residues that properly connect BTK and SYK with PLC γ 2 and initiate a cascade of intracellular downstream signaling events [251, 252]. Moreover, it has been reported that the transcription factor Pax5 is necessary for

the regulation of BLNK expression [253]. Furthermore, interaction of BLNK with VAV and NCK correlates with cytoskeletal organization in B cells [250, 251].

As a result of aberrant signaling in BLNK-deficient mice, both physiological as well as pathophysiological role(s) of BLNK have been suggested [51]. Lack of BLNK expression in mice results in partial block of pre-B cell, lack of peripheral B1 B cells, reduced numbers of B2 cells and development of pre-B cell lymphoma [33, 34, 51, 254]. It has been shown that malfunction of BLNK leads to immunodeficiency as well as a reduction in the activation of PLC γ 2 [51, 255]. Moreover, 50% of pre-B ALL in childhood is caused by defective BLNK expression, suggesting that BLNK plays a key role in pre- B cell differentiation and tumor suppression [32].

Structurally, BLNK consists of highly conserved N-terminal leucine-zipper motif important for its membrane tethering, followed by an N-terminal region containing tyrosines in YxxP formula, a proline-rich domain and a C-terminal SH2 domain [51, 256, 257]. BLNK serves as an adaptor molecule to assemble various components in the form of signalosome complex to regulate signal transduction via inducible docking sites of the phosphotyrosine N-terminus domain, proline rich domain and SH2 domain, such as HPK1, SYK, BNAS2, Growth factor receptor-bound protein 2 (Grb2), VAV and NCK (Y72) PLC γ 2 (Y84, Y178, Y189), BTK (Y96) [255, 258-261] (Figure 8). This, in turn, couples the BCR to the activation of, among others, the PLC γ /PKC, PI3K/AKT, NF κ B and RAS/RAF/ERK signaling pathways [125-129]. Moreover, BLNK can bind to phospho-tyrosine 204 (pY204) in the ITAM region of Ig α for its activation [262].

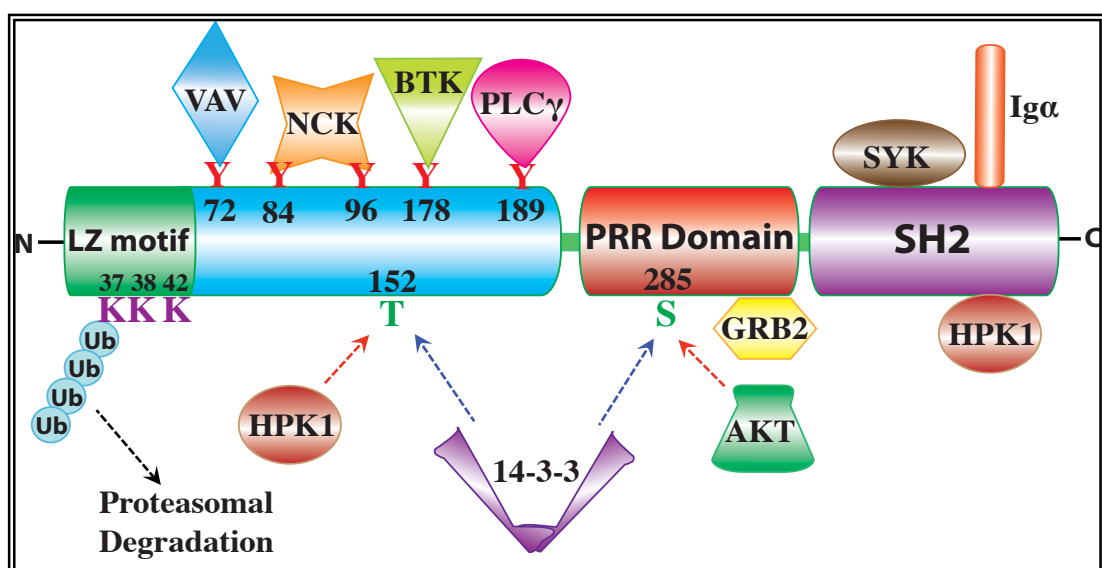


Figure 8. Schematic representation of BLNK domain structure showing regulatory phosphorylation/degradation residues as well as interacting protein partners.

According to a recent study, HPK1 phosphorylates BLNK at threonine 152 (T152) thereby creating a binding site for 14-3-3 proteins [263] that facilitates ubiquitination, and proteasomal degradation of BLNK [263] (Figure 8). Different modes of interactions have been suggested with respect to the translocation of BLNK to the plasma membrane and the triggering of B cell activation. It has been reported that BLNK requires a constitutive association with the CBL-interacting protein of 85 kD (CIN85) to control their subcellular location [264]. Also a putative leucine zipper motif in the N-terminal basic domain of BLNK could be important for membrane binding [257]. Furthermore, it has been proposed that CMTM7 acts as a transmembrane linker of BLNK and BCR [265]. In the current study (Paper IV), we have identified that 14-3-3 is an important regulator for binding of BLNK with SYK and controlling BCR downstream signalosome (Mohammad, et al., 2015).

1.7.4 Karyopherins (Importins)

Karyopherins are a group of adaptor molecules responsible for transporting target proteins. Karyopherins comprise of Importins [266] and Exportins [267, 268], which mediate nuclear import and export, respectively. The most classical nuclear protein import pathways are mediated by Importin β and Importin α , which are also known as Karyopherin β 1 (KPNB1) and Karyopherin α (KPNA) respectively. In this classical pathway many cargoes bind directly to Importin α , which serves as an adaptor protein to tether the cargo to Importin β forming a trimeric transport complex that moves through the nuclear pore into the nucleus. Importin β is composed of 19 HEAT repeats, structural motifs consisting of two antiparallel helices connected by a short loop [269]. These HEAT repeats are stacked to form superhelical structures [270]. The HEAT repeats (1-8), (3-8) and (7-19) in Importin β are important for RanGTP, nuclear pore complex (NPC) and Cargo/Importin α binding (Figure 9A). The number of Importin β subunits differs between organisms. The genome of *Saccharomyces cerevisiae* encodes for 14 Importin β subunits, whereas the genome of human encodes for 20 Importin β subunits, of which 10 are Importins and 7 are exportins [271]. Importin α subunits are composed of a flexible N-terminal Importin β binding (IBB) domain, ten armadillo (ARM) motifs, and an acidic C-terminal domain (Figure 9B). The IBB domain of Importin α serves as the binding site for Importin β .

The classical nuclear import cycle is the most used pathway for nucleocytoplasmic transport inside cells. In this cycling, cargo proteins are recognized by Importin α , which links the target proteins to Importin β [272]. A trimeric complex is formed and translocation into the nucleus occurs via Importin β -mediated association with the nuclear pore. In the nucleus, the trimeric complex dissociates as a result of a conformational change of Importin β after binding of RanGTP, which results in the release of the cargo-Importin α

complex [273]. Next, the auto-inhibitory region on the IBB of Importin α , the Cse1 and Nup2 facilitate delivery of the target protein into the nucleus [274-279]. To fulfill another round of cargo import, Importin α is recycled back to the cytoplasm with help of the export receptor in complex with Ran/GTP, Cse1/RanGTP (Figure 10) [280, 281]. Thus, Importin β -like proteins are able to translocate target proteins, using different modes, such as direct canonical and NLS-dependent or direct non-canonical NLS-independent cargo binding and form a complex with Importin α or with other β -like Importins.

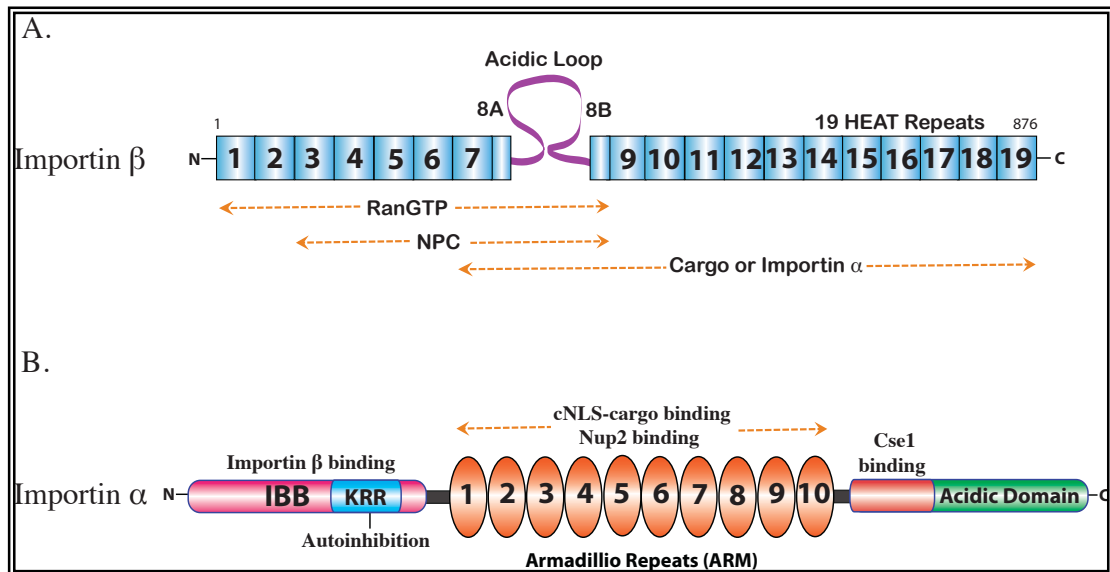


Figure 9. Domain structure of Importin β (A) and Importin α (B).

Importin-7, also called (Ran-binding protein 7), a 120 KDa protein of 1036 amino acids is a cytoplasmic and nuclear protein that is ubiquitously expressed in most tissues [282]. Importin-7 functions in the nuclear transport of target proteins as a nuclear transport receptor or a scaffold protein in association with other Importins, such as Importin 3 [283, 284]. Importantly, increase in the steady state of Importin 7 has been detected in different cancer cells, such as colorectal carcinoma, breast and ovarian tumors [285, 286], which might be a good candidate for drug improvement. Importin-7 is considered to act as receptor for proteins harboring NLS to facilitate their translocation via the NPC by Ran-dependent mechanism [287].

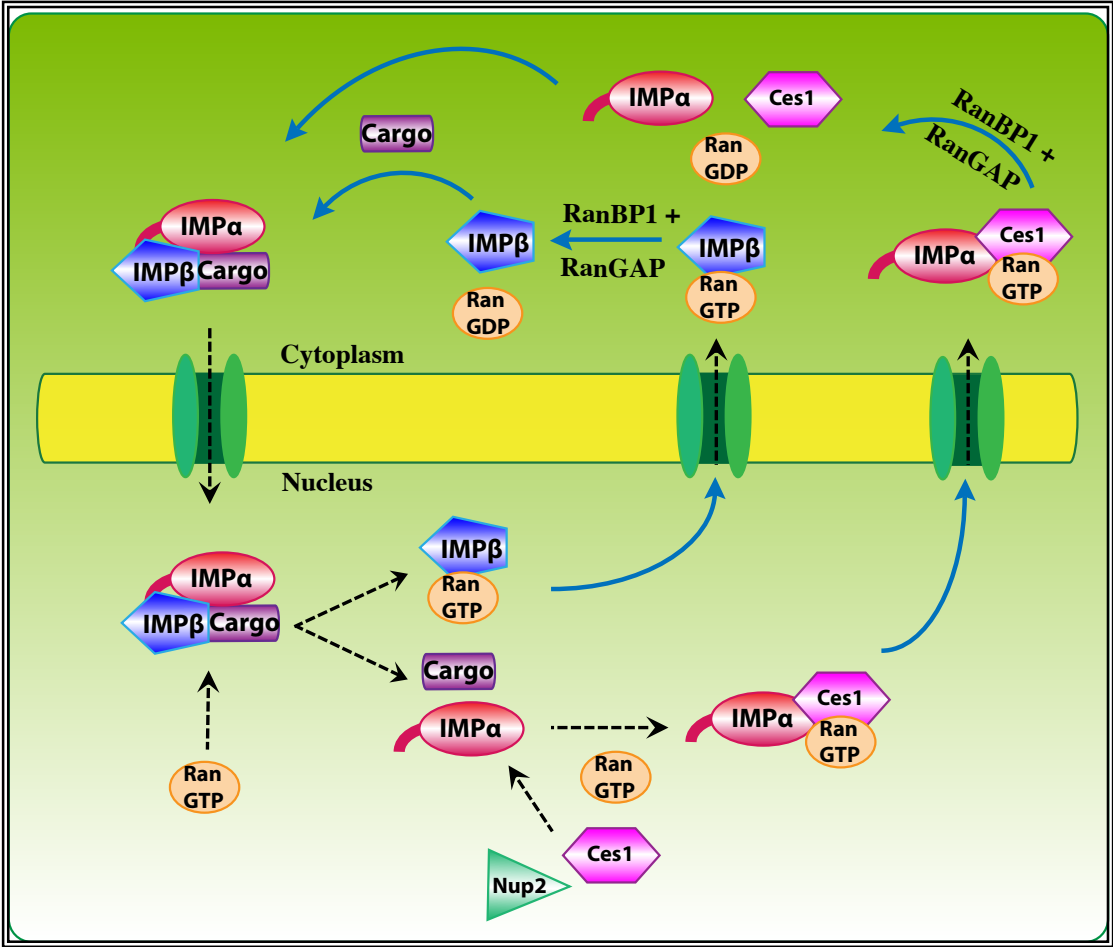


Figure 10. A schematic overview of the Importin β and Importin α nuclear import and export cycles.

2 AIMS

2.1 GENERAL AIMS

The overall aim of this thesis was to investigate alterations in the signalosome assembly downstream of the BCR signaling following stimulation of this receptor. In particular, the role of AKT and 14-3-3 in the regulation of this signalosome complex down stream of BCR signaling in B cells was investigated. Our aim was to get insight into the molecular mechanisms responsible for these regulations and explore new pathways that could enable us understand the consequences of the B cell malignancies that may contribute to the development of new treatment strategies.

2.2 SPECIFIC AIMS

- To study the role of AKT and 14-3-3 in the regulation of intracellular signaling of BTK in B cells.
- To investigate the role of ANKRD54 (LIAR) in the nucleo/cytoplasmic shuttling of BTK.
- To characterize newly identified protein-binding partners downstream of AKT-mTORC1/2 signaling.
- To explore the potential role of AKT, 14-3-3 and Importin 7 in the regulation and subcellular localization of BLNK and SYK.

3 MATERIALS AND METHODS

3.1 CELL LINES

Namalwa (human Burkitt lymphoma B-cells), K562 (human chronic myelogenous leukemia cells), A20 (mouse B-lymphoma), Nalm-6 (pre-B-cell leukemia), RBL- 2H3 (rat basophil leukemia cells with mast cell characteristics), Jurkat (human T-lymphocyte), Phoenix GP and PG13 (retrovirus producer lines based on human embryonic kidney HEK293T cells), and NIH 3T3 (mouse embryonic fibroblast) and Cos-7 (African green monkey fibroblast-like kidney) cell lines were obtained from the American Type Culture Collection (ATCC). All hematopoietic cell lines were cultured in RPMI1640 medium supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) (Life technologies). The adherent cell lines were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated FBS. All cell lines were cultured at 37 °C in a humidified 5% CO₂ incubator.

3.2 MASS SPECTROMETRIC ANALYSIS

Mass spectrometry (MS) is a powerful chemical analytical technique that requires high-energy electrons for breaking a protein into small peptides. The peptides are further breaking down into ions, which basis on the measurement of the atomic mass of each molecule [288]. MS is used to identify the chemical structures of the unknown proteins isolated from natural sources. MS is also combined with chromatographic separation methods for detection of proteins in a mixture [289]. Here, in this study, Namalwa cells were used for MS analysis after specific treatment according to the purpose of the project. Namalwa cells were lysed with lysis buffer and incubated on ice for 20 min, with intermittent mixing. For pre-clearing, protein A and G sepharose beads and agarose anti-IgG were added to each tube. We incubated the samples by rotating for 60 min at 4°C, followed by centrifugation (3000 rpm) at 4°C for 10 min. The supernatants were filtered to new tubes containing 50 µl of Flag M2 agarose beads or Dynabeads (Life Technologies). The samples were incubated under rotating conditions at 4°C. The beads/Dynabeads were washed several times with PBS, using centrifugation or magnetic beads, when using Dynabeads. The beads/Dynabeads were subsequently washed three times with cold Flag-rinsing buffer. The purified protein complexes on the anti-Flag M2 agarose beads or Dynabeads were gently eluted with 2% NH₄OH and lyophilized, and prepared for gel-free mass spectrometry analysis. Samples were digested in solution and analyzed by liquid chromatography-MS using high performance liquid chromatography (HPLC) system coupled to an LTQ-MS or a capillary HPLC system combined to an LTQ-Orbitrap-MS. The resulting MS/MS spectra were analyzed using the MASCOT program against the human Ensembl Database release. To biochemically validate the proteins identified by MS/MS data, co-

immunoprecipitation experiments, western blot analysis and Immunofluorescence assays were performed.

3.3 TRANSFECTION METHODS

3.3.1 RNA interference

Small interfering RNAs (siRNAs) are major effectors in the RNA interference (RNAi) pathway, comprising double-stranded RNA molecules (20-25 base pairs in length) that prevent gene expression, primarily by inducing degradation of specific messenger RNA (mRNA). For transfection of siRNA, 100 nM of 14-3-3 siRNA (Santa Cruz) or a “scrambled” sequence with no significant homology to any known gene sequences was used. The siRNA was diluted in a Buffer R (Neon electroporation Kit; Invitrogen, Carlsbad, CA) and added to 1.5 million Namalwa cells in a final volume of 10 μ l per reaction. The transfection was carried out using the Neon electroporation system according to the manufacturer’s instruction. Cos-7 cells were also used for knockdown assay, using Lipofectamine 2000, (Life technologies), the procedure of 14-3-3 siRNA was performed according the manufacturers protocol. 48 h post transfection, the cells were harvested and following lysis processed for western blotting analysis.

3.3.2 Plasmid transfection

For adherent cells, transient transfections of the plasmids encoding specific proteins were performed in 6-well plates using the cationic polymer polyethyleneimine (PEI) (Polysciences Inc. USA), according to the manufacturer’s protocol.

3.4 PROTEIN ANALYSIS

3.4.1 Immunoprecipitation (IP)

Immunoprecipitation is one of the methods used for protein precipitation and purification. The principle of an IP is based on using a specific antibody against a target protein forming an immune complex. This complex can then be captured on beads onto Protein A or G. Following IP, the protein partners are analyzed using Western blotting. IP analysis was performed using Dynabeads protein G (Life Technologies) according to the manufacturer’s protocol. Whole cell lysates were incubated with the indicated antibodies and rotated at 4 °C for 2 h. To co-IP the antibody-antigen complex, 50 μ l of protein G Dynabeads were added and the mixture was incubated by continuously rotating at 4 °C for 1 h. The Dynabeads were washed three times with PBS buffer, suspended in sample buffer, boiled at 65 °C for 4 minutes and the resultant analyzed by sodium dodecyl sulphate, SDS-PAGE. Negative controls for co-IPs were lysates

prepared under the same conditions with the addition of a control antibody (anti-HA).

3.4.2 Western blotting

Western blot (WB) or immunoblot is an important analytical technique used in cell and molecular biology research for detecting proteins in phosphorylated or native states. By using a WB, investigators are capable of distinguishing specific proteins from a complex mixture of proteins isolated from cells. The Western blot method was developed in the laboratory of Harry Towbin [290]. To facilitate lysis of cells, various detergents, buffers and salts can be added. Using a cocktail of phosphatase and protease inhibitors can inhibit degradation and dephosphorylation of sample proteins. Gel electrophoresis is usually used in order to separate the macromolecules. To facilitate antibody detection of the specific protein, the proteins are transferred from the gel and blotted onto a nitrocellulose membrane. To reduce non-specific binding, the membrane is blocking with a 5% bovine serum albumin (BSA) or LICOR-Blocking buffer depending on the specificity of the primary antibody. After blocking, a dilute solution of primary antibody is incubated with the membrane under gentle agitation. Following incubation of the primary antibody, the membrane is extensively washed (to remove unbound primary antibody), and further incubated with a LICOR secondary antibody (mentioned before). Finally, the membrane is again washed to remove unbound antibody and the protein of interest is detected using Odyssey infrared imaging system (Li-COR Biosciences GmbH, USA).

3.5 MICROSCOPY

3.5.1 Immunocytochemistry

Immunocytochemistry is a frequently used laboratory technique that is used to anatomically identify expression and localization of a specific protein in cellular compartments with the help of a specific antibody recognizing the protein of interest. 16 h after seeding, cells were transfected with plasmids or siRNA and left to grow for an additional 48 h. For immunostaining, cells were washed several times with PBS and fixed with 2.5% formaldehyde for 15 min at room temperature. The fixed cells were subsequently permeabilized using 0.1% Triton X-100 in PBS for 15 min, blocked in 0.1% c-BSA for 1 h at room temperature and following addition of primary antibodies, incubated overnight at 4°C. The cells were washed and further stained with a Cy3-conjugated goat anti-mouse IgG (1:500) or with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:500). Finally, the cells were incubated with DAPI 5µg/ml DAPI diluted in PBS (4=6-diamidino-2-phenylindole, dihydrochloride) (Molecular Probes) to stain cell nucleus and then intensively washed prior to imaging.

3.5.2 Confocal microscopy

To obtain high-resolution optical images of the cells, images were captured on a Leica DMRXA confocal microscope equipped with a 3D digital microscopy workstation (©Leica Microsystems, Wetzlar, Germany). Captured images were processed and analyzed using slide book (Intelligent imaging Innovations, Inc. Denver, Colorado, USA).

3.5.3 Fluorescence microscopy

Following the immunocytochemistry technique, co-localization of the indicated proteins was monitored using fluorescence microscopy. Images were captured using Olympus microscope (Olympus-IX81). For image processing, the cellSense Dimension software (Olympus, Tokyo, Japan) was used.

3.6 CELL PROLIFERATION AND VIABILITY ASSAY

Assays to measure cell proliferation, viability, and cytotoxicity are normally used to monitor the response of cells in culture following treatment with various drugs. Various assays are currently available for determining cell proliferation. These assays, which can only estimate cell number, are based on measurements related to metabolic activity (MTT or Alamar blue). In our study, we used a cell proliferation assay, which is capable of monitoring the number of cells over time as well as the number of cellular divisions. The Countess™ automated cell counter from Life Technologies using the viability trypan blue dye can provide both the rate of proliferation as well as the percentage of viable cells.

3.7 NUCLEAR AND CYTOPLASMIC FRACTIONATION

To determine the subcellular localization of different proteins inside the cellular compartments, cytoplasmic and nuclear extracts are used instead of whole cell lysates. In our studies, we used NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce), which allows a stepwise lysis of cells that generates both functional cytoplasmic and nuclear protein fractions. Namalwa nuclear and cytoplasmic extracts were prepared according to the manufacturer's protocol.

3.8 IN VITRO KINASE ASSAY

In vitro kinase assay is a specific method used to measure the enzymatic activity of protein kinases. To achieve this, an antibody specific to the kinase is added to the cell lysate in one side and the specific antibody targeting the

expecting substrate to another cell lysate will be added. The antibody will bind to the desired target kinase and the target protein. The mixture is incubated with the protein G Dynabeads separately. Then the Dynabeads from both mixtures are incubated in the presence or absence of ATP (200 μ M) in a kinase reaction buffer for 30 min at 30 °C. After the incubation, the reactions were stopped by addition of sample buffer and the proteins were run on 4-12% Bis-Tris SDS-PAGE gel and phosphorylation visualized by Western blot analysis. If the substrate was phosphorylated by the kinase, it appears on the membrane. If the kinase was inactive or was unable to phosphorylate the chosen substrate, the gel remains blank. The kinase assays can be run on recombinant kinases, or cloned and purified from a protein expression system. They don't necessarily need to be isolated from the host cells. In our study immunoprecipitated AKT from Namalwa cells was incubated with immunoprecipitated BLNK or SYK and *in vitro* kinase assay was performed.

4 RESULTS AND DISCUSSIONS

4.1 PAPER I

Dual Phosphorylation of BTK By AKT/Protein Kinase B Provides Docking for 14-3-3 ζ , Regulates Shuttling, and Attenuates both Tonic and Induced Signaling in B Cells

In this work, we studied the role of 14-3-3 protein, as a novel BTK-interacting partner. We undertook a mass spectrometry approach and proteomics analysis to dissect the BTK signalosome complex and identify novel BTK-interacting proteins. We performed the proteomic analysis using the hematopoietic cell line, Namalwa, as described in our recent papers [231, 241]. After analyzing the MS/MS data of the highest peptide coverage, we detected the presence of 14-3-3 isoforms (zeta 37.5% and gamma 28.3%) as BTK binding partner proteins. To biochemically confirm this finding, we carried out western blotting analysis of the BTK signalosome complex and identified 14-3-3 ζ protein, as one of the novel interacting partners for BTK *in vivo* (of B-cell lines and of primary B-lymphocytes). The 14-3-3 ζ -protein family is known to bind serine/threonine-phosphorylated proteins [212, 291]. Affinity purifications have revealed hundreds of interacting partners for the 14-3-3 in humans [292]. Notably, a novel consensus motif (RXRXXpT/S) known as a target site for AKT/PKB phosphorylation was important for the analysis, since this motif also functioned as a binding site for 14-3-3 ζ [293]. By careful inspection of the BTK sequence, we found two AKT/PKB consensus sites, one located in the PH domain and the other in the kinase domain. Indeed, the motifs (numbered after the BTK sequence) ⁴⁶RGRRGpS⁻⁵¹ and ⁴⁹⁰RHRFQpT⁻⁴⁹⁵ are the perfect matches of the AKT target sites; therefore we focused on these two motifs, which we considered potentially important as 14-3-3 ζ binding-sites. Here, we identified that the corresponding sites in BTK were phosphorylated by the AKT/PKB that facilitates interaction with 14-3-3 ζ through a phosphorylation dependent mechanism. Furthermore, we identified from the sequence alignment that the 14-3-3 binding region in BTK is highly conserved across species.

Our previous studies have shown that BTK is mainly cytoplasmic and redistributes to the inner cytoplasmic membrane upon PI3K activation [294]. On the other hand, the nucleocytoplasmic shuttling of 14-3-3 and BTK has been shown in separate studies, but the shuttling mechanism and the functional consequences remain elusive for both proteins [216, 295]. Therefore, it was tempting to see if 14-3-3 can regulate the subcellular localization of BTK. Interestingly, siRNA knockdown of 14-3-3 ζ increased the nuclear translocation of BTK, while overexpression of 14-3-3 ζ resulted in accumulation of BTK in the perinuclear region.

In addition, whereas the S51A/T495A-BTK mutant failed to bind 14-3-3 ζ , the phosphomimetic mutant S51D/T495D-BTK enhanced the interaction capacity with 14-3-3 ζ upon treatment with the proteasome inhibitor (MG132). The PI3K inhibitor (LY294002) and/or dominant negative AKT (AKT-DN) blocked BTK-serine/threonine phosphorylation and abolished BTK interaction with 14-3-3 ζ . Moreover, two newly characterized inhibitors 14-3-3 inhibitor (BV02) [296] and Ibrutinib (PCI-32765) inhibitor [297] interrupted binding of 14-3-3 to BTK. Remarkably, S51D/T495D-BTK mutant displayed enhanced tyrosine phosphorylation and strong binding to 14-3-3 ζ , which subsequently induces BTK ubiquitination and degradation thereby attenuating the BCR-signaling pathway. In stark contrast, the S51A/T495A mutant completely failed to become phosphorylated and was not at all ubiquitinated (Figure 11). Although cell membrane tethering of the PH domain is key for the activation of BTK, nuclear-localized BTK has been reported [298]. In previous studies, we and other groups have shown several proteins that negatively regulate BTK, including peptidylprolyl cis/trans isomerase Pin1, Caveolin-1 and PKC β [86, 95, 96]. Through my work, we now also identify another kinase AKT as a novel negative regulator of BTK, through phosphorylation on two sites that facilitates its interaction with 14-3-3 ζ , which contributes to the termination of B-cell receptor signaling.

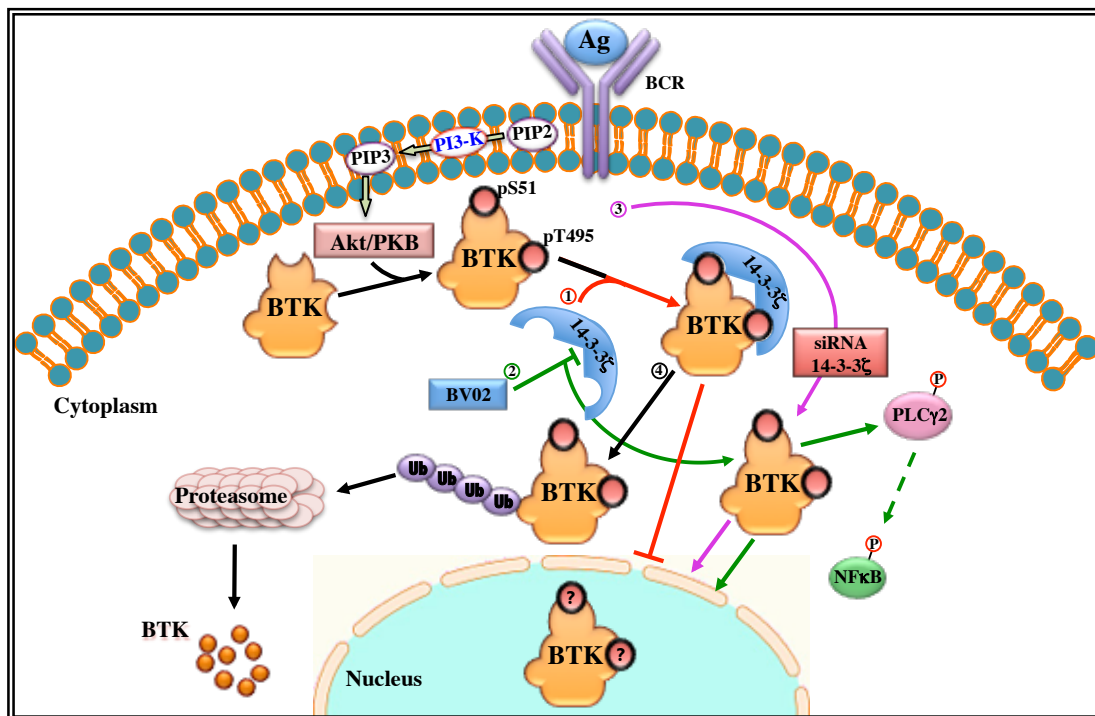


Figure 11. Schematic view of the BTK signalosome complex regulation by 14-3-3 protein. PI3K-mediated activation of AKT/PKB leads to phosphorylation of BTK at S51 and T495. Subsequently, 14-3-3 ζ interacts with phospho-BTK-S51/T495 and prevents translocation of BTK to the nucleus. Moreover, binding of 14-3-3 ζ to activated BTK stimulates ubiquitination and degradation of active BTK, leading to the termination of BCR signaling.

4.2 PAPER II

Regulation of Nucleocytoplasmic Shuttling of Bruton's Tyrosine Kinase (BTK) through a Novel SH3-Dependent Interaction with Ankyrin Repeat Domain 54 (ANKRD54)

In this study, the BTK signalosome assembly was the main focus to study interactome of BCR under starved, activated and Btk-inhibited conditions. In this work, we generated a stably Flag-tagged BTK expressing Namalwa B-cell line and Flag-affinity purification was used to capture signalosome complexes, followed by top-down mass spectrometry approach is applied on gel-free samples in order to identify novel BTK-interacting proteins. After proteomics analysis of the MS-MS data, we selected a novel interactor with BTK, an ANKRD54, which we found specifically influences nuclear shuttling of BTK [241]. Moreover, we further characterized the interaction between BTK and ANKRD54 using biochemical analysis and also by subcellular localization using confocal microscopy. Interestingly, we found that ANKRD54 potently sequesters BTK in the cytoplasm. In a similar fashion, TXK (RLK), another TFK, was also excluded from the nucleus in the presence of ANKRD54. During our analysis of ANKRD54, murine LIAR (LYN-interacting ankyrin repeat protein- ANKRD54) was published as a novel Lyn-binding protein through the SH3-domain and forming a multiprotein complex and influencing erythropoietin-induced differentiation of erythrocytes [243]. We also found that ANKRD54 modulates nuclear localization of BTK in an SH3-dependent manner, while two other nuclear-resident proteins, estrogen receptor beta (ER β) and transcription factor T-bet (T-Box expressed in T cells) were unaffected. A peptide consisting of 22 amino acids from the C-terminus of the BTK SH3 domain (ARDKNGQEGYIPSNYVTE-AEDS) was sufficient for the pull-down of endogenous ANKRD54.

In a previous study, we also observed that the SH3-domain is a negative regulator of the nuclear shuttling of BTK, since a BTK- Δ SH3 mutant predominantly localized to the nucleus at steady-state conditions [298]. In this work, we observed that green fluorescent protein-tagged (GFP)-BTK- Δ SH3-NLS, behaved similarly to the GFP-BTK-NLS fusion in being exclusively nuclear. Importantly, ANKRD54 failed to interact with GFP-BTK- Δ SH3-NLS protein and prevent it from entering the nucleus. In contrast, in the presence of ANKRD54, the GFP-BTK-NLS protein became completely excluded from the nucleus. Also, we showed that the efficacy of cytoplasmic retention of GFP-BTK-NLS, but not that of the GFP-BTK- Δ SH3-NLS fusion, by ANKRD54 is compromised in the presence of overexpressed BTK-SH3 domain. In addition, active BTK is required for the *in vivo* tyrosine phosphorylation of ANKRD54, suggesting that ANKRD54 may be a direct substrate of BTK. Collectively, these data suggest that the SH3 domain of BTK is crucial for ANKRD54

interaction, resulting in BTK retention in the cytoplasm. Our results show a novel and potentially important partner for BTK in B-lymphocytes, and that ANKRD54 controls BTK shuttling into the nucleus in an SH3-dependent manner (Figure 12).

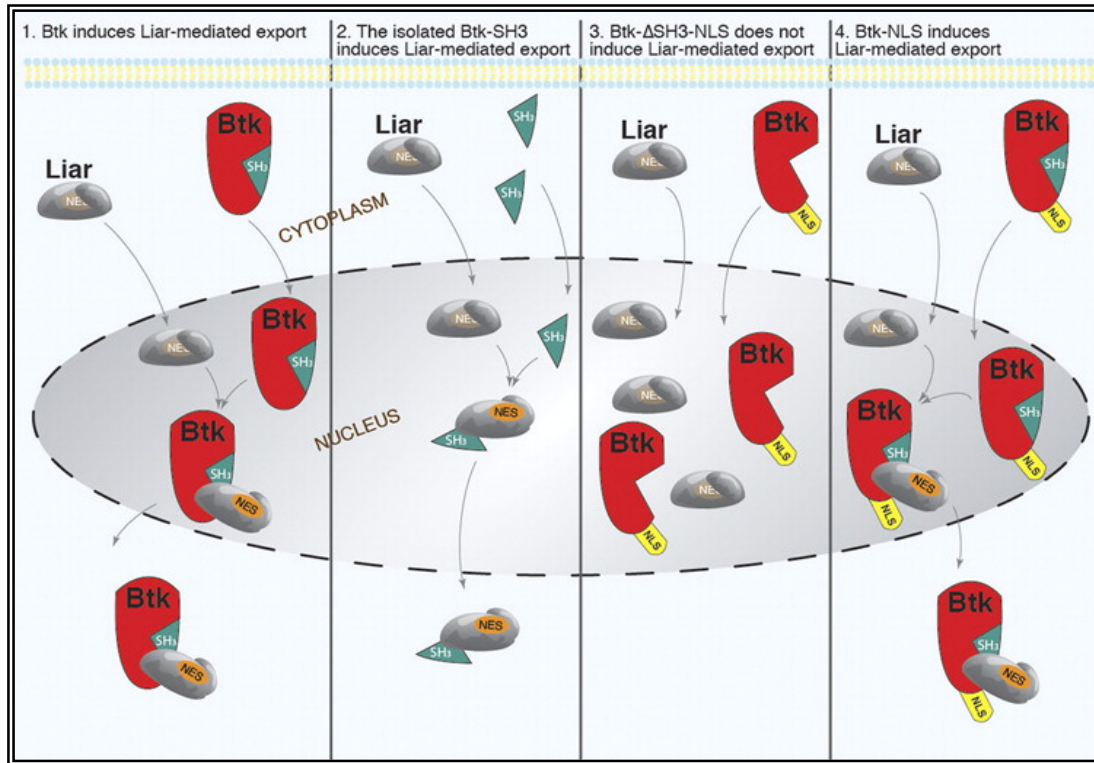


Figure 12. Graphic model of the BTK nucleocytoplasmic shuttling mediated by the SH3-domain dependent interaction with LIAR (ANKRD54) [241].

4.3 PAPER III

B Cell Receptor (BCR) Activation Predominantly Regulates AKT Associates Motifs Phosphorylation in Proteins Related to RNA Processing

In the present study, we employed affinity purification of endogenous proteins harboring RXXRXXpS/T motif, as an mTORC2/AKT consensus substrates, towards pull down of complex proteins in Namalwa B cells. To identify novel phosphorylated proteins containing the AKT consensus motif, we performed proteomic analysis on Namalwa cells using mass spectrometry (MS/MS) technique. The peptide coverage for the 446 novel proteins found in the proteomic data was obtained following Mascot prediction scoring. To biochemically confirm the data of the MS/MS analysis, immunoprecipitated enrichment of endogenous proteins containing AKT consensus motif were resolved on SDS-PAGE and immunoblotted with phospho-specific (RXXRXXpS/T) motif antibody, as an AKT consensus substrate. To further verify the proteomics data, we investigated the phosphorylation of two of the identified proteins Myocyte-specific enhancer factor 2D (MEF2D) and RNA-binding motif 25 (RBM25) in Namalwa cells. Immunoprecipitation with anti-MEF-2D and RBM25 antibody resulted in the pull-down of endogenous MEF-2D and RBM25 followed by immunoblotting with anti-RXXRXXpS/T demonstrated positively the phosphorylation of both proteins on the AKT-consensus motif, suggesting that these proteins are downstream substrates of the mTORC2/AKT pathway.

Using MS/MS raw-data, we performed a detailed bioinformatics analysis and identified at least 186 proteins containing the RXXRXXS/T consensus motif. In addition, we also found the 260 proteins, which lack a canonical AKT motif, but were present in the complex. Moreover, we detected only 85 proteins that were up regulated, while 277 proteins were down regulated following anti-IgM stimulation. With respect to the up regulated proteins, a group of ribosomal proteins were overrepresented. Another group that seems to be affected are proteins, which regulate the cell cycle through various mechanisms, including DNA binding and transcriptional regulation factor proteins. Other ribosomal proteins were enriched among the down regulated proteins, as were proteins involved in ribosome biogenesis. The largest group found to be down regulated in the phosphoproteome, consisting of proteins related to RNA binding and splicing and mRNP export. These proteins are most likely components of the late spliceosomal complexes linked with mRNP export, as described in a previous study [299].

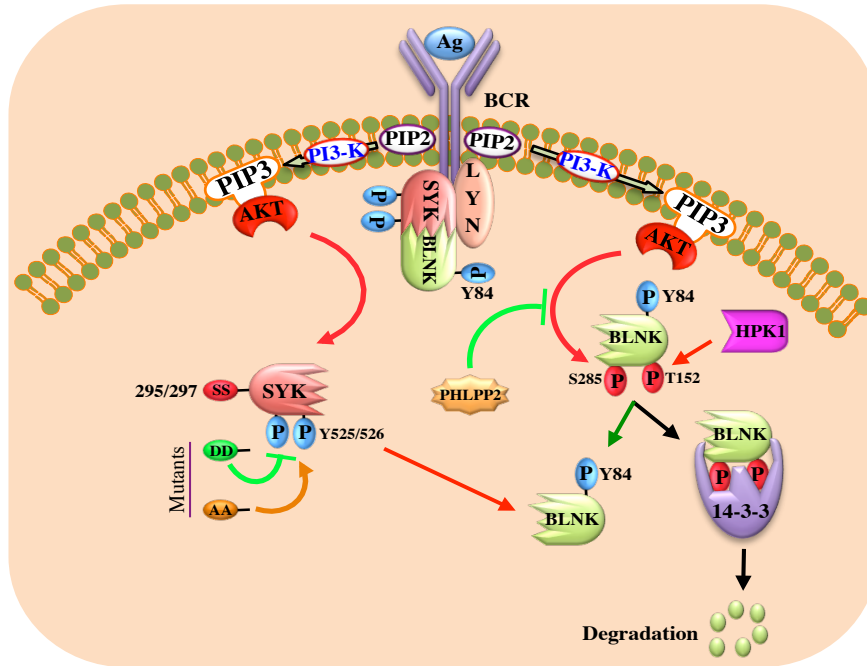
4.4 PAPER IV

AKT/PKB Attenuates SYK and BLNK through 14-3-3 Impairing Nuclear Translocation of SYK via Importin 7 in B Cells

In our recent study, we have characterized the role of AKT/PKB on the phosphorylation status of BTK that facilitates the docking of 14-3-3 ζ [231]. 14-3-3 proteins interact with a plethora of signaling proteins to fine-tune the BCR activation, thereby controlling cell proliferation, differentiation and/or apoptosis. Therefore, we sought to study the proteomics of AKT consensus motif RXRXXS/T following BCR engagement. The mass spectrometry (MS/MS) data and analysis of protein sequence using Scansite 3 program (<http://scansite3.mit.edu/>) identified BLNK and SYK as target candidate sensitive for AKT activity. Although SYK and BLNK are known as proximal signal transducer elements of the BCR, little is known about the molecular mechanisms underlying 14-3-3 interactions with BCR downstream signalosome.

Here, we describe that the adaptor protein 14-3-3 interacts with SYK and BLNK in a Ser/Thr phosphorylation-dependent manner. We found that AKT/PKB induces BLNK and SYK Ser/Thr phosphorylation, promoting their association with 14-3-3. Moreover, the AKT/PKB inhibitor MK-2206 reduces the phosphorylation of BLNK and SYK. In addition, 14-3-3 is required for the stable interaction between SYK and BLNK. Furthermore, 14-3-3 attenuates SYK binding to Importin 7 and thereby abrogates shuttling to the nucleus. The mechanism of nuclear translocation of SYK is not known. In a previous study, it was suggested that the unconventional shuttling sequence present in SYK is critical for subcellular localization of SYK [300]. To understand the biological role of 14-3-3 on SYK translocation to the nucleus, we treated Namalwa cells with BV02, a novel 14-3-3 inhibitor and show that 14-3-3 altered the subcellular localization of SYK. To further explore the consequences of the 14-3-3 and SYK interaction on the translocation of SYK to the nucleus, we pre-treated Namalwa cells with BV02 and subsequent co-immunoprecipitation analysis revealed that inhibition of 14-3-3 activity using BV02, resulted in the increased interaction of SYK with Importin 7. Here, for the first time we report a novel association of Importin 7 with SYK that modulates the nuclear shuttling of SYK. Collectively, these results suggest that 14-3-3 plays an important role in the nucleocytoplasmic shuttling of SYK by controlling the stable interaction between BLNK and Importin 7 (Figure 13).

A.



B.

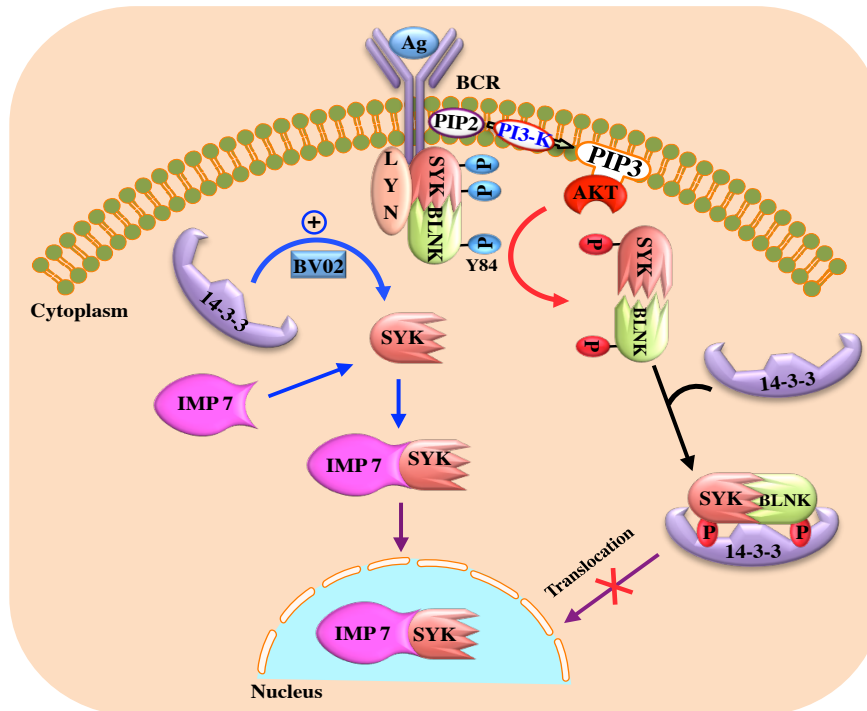


Figure 13. A model illustrating the regulation of BLNK and SYK function by AKT and 14-3-3. (A). AKT phosphorylates BLNK and SYK in the cytoplasm leading to the recruitment of 14-3-3. Furthermore, 14-3-3 binding to SYK interferes with its interaction with Importin 7 impeding translocation of SYK to the nucleus. Therefore, for the nuclear translocation of SYK, de-assembly of the SYK/BLNK/14-3-3 complex is required. (B). AKT phosphorylation sites in SYK are crucial for the modulation of SYK phosphorylation at Y525/526, as well as phosphorylation of BLNK at residue Y84. Notably, AKT and HPK1 phosphorylate BLNK at S285 and T152, respectively, thereby attenuating the activity and signaling of BLNK.

5 CONCLUSIONS

The studies in this thesis have shown that:

Paper I:

- AKT/PKB is the serine/threonine kinase responsible for the phosphorylation of BTK on two sites (Ser51 and Thr495).
- 14-3-3 is a novel interacting protein partner for BTK.

Paper II:

- ANKRD54 (LIAR) specifically influences nucleocytoplasmic shuttling of BTK and the association is mediated through the SH3 domain.
- The interaction between BTK and ANKRD54 (LIAR) is independent of the BTK kinase activity.

Paper III:

- MEF2D and RBM25 were identified as novel AKT-mTORC1/2-induced phosphorylation substrates following B cell receptor activation.
- BCR activation causes selective up- and down regulation of mTORC2/AKT target sites related to ribosome biogenesis and RNA-processing.

Paper IV:

- AKT/PKB induces BLNK and SYK Ser/Thr phosphorylation and facilitates docking sites for 14-3-3 and Importin 7 interaction, thereby regulating translocation of SYK to the nucleus.
- AKT phosphorylation sites (Ser295/Ser297) in SYK are important for controlling SYK Y525/526 phosphorylation.
- BLNK phospho-tyrosine 84 (pY84) correlates with the SYK phospho-tyrosine 525/526 level.

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